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EMBRYOLOGIA, Vol. 5, No. 1, pp. 1-7 (April, 1960)

CYCLIC CHANGES OF THE CORTEX AND THE CYTOPLASM OF THE FERTILIZED AND THE ACTIVATED SEA URCHIN EGG*

I. CHANGES IN THE THICKNESS OF THE HYALINE LAYER

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The contribution to our knowledge of the cyclic changes in the egg cortex after fertilization and parthenogenetic activation was made first by HERLANT (1920). Since that time many investigations have been performed on the cyclic changes both in the cytoplasm and in the cortex of fertilized and activated sea urchin eggs (cf. ROTHSCHILD, 1956). Among them, the papers of MONROY and MONTALENTI (1947), ZEUTHEN (1951), and SWANN and MITCHISON (1953) are considered to be most important.

MONROY and MONTALENTI found that the mode of plasmolysis of the egg and the birefringence of the cortical layer cyclically change not only in normal eggs but also in colchicine-treated ones, in which the aster formation is suppressed, and they concluded that these cyclic changes are independent of the cycles in aster formation.

According to ZEUTHEN (1951), on the other hand, even when fertilized eggs are treated with colchicine, rhythmical changes in their oxygen consumption are observed, which is synchronized with a cycle of dissolution and reappearance of the nuclear membrane. Further, SWANN and MITCHISON (1953), reported that cyclic changes in the rigidity and light-scattering properties of the cortex are associated with cycles of the chromosome condensation which persists in colchicine-treated eggs, in which the aster formation and cleavage are prevented.

From the above-mentioned investigations, it may be postulated

* Some of the results of this paper have been reported briefly in Japanese (KUNO, 1954).

that some of the cyclic changes observed in the cortex and the cytoplasm of fertilized and activated eggs are independent of the aster formation, though they have some relation to the nuclear activity, such as the chromosome condensation.

In 1952, DAN and ONO found that the hyaline layer is suddenly lifted off the egg surface a few minutes before the onset of cleavage. The present work was undertaken to study the relation between this lifting of the hyaline layer and the nuclear activities. The former will be called the thickening of the hyaline layer for the convenience of description in the present paper, though, according to DAN and ONO, the apparent thickening of the layer is really due to an accumulation of some fluid between the layer and the egg surface.

MATERIAL AND METHODS

Eggs of the following four sea urchins were employed: *Clypeaster japonica* available at Misaki, and *Temnopleurus toreumaticus*, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* at Sugashima.

6 per cent butyric acid sea water (6 cc. of N/10 butyric acid+94 cc. of ordinary sea water) and 30 per cent thymol sea water (30 cc. of saturated thymol solution in distilled water+70 cc. of ordinary sea water) were used as the activating agents. The eggs were exposed either to butyric acid sea water for 40-60 seconds, or to thymol sea water for 30 seconds, and were then returned to ordinary sea water.

In a series of experiments, the unfertilized egg was divided into two fragments by strong centrifugation at 10,000–15,000 *g* for 10–15 minutes, and both the centrifugal non-nucleated and the centripetal nucleated fragments were used as materials.

EXPERIMENTAL RESULTS

1. Activation of whole eggs

Clypeaster eggs which had been activated with butryic acid were observed at 15 minutes' intervals for 3 hours following the activation. An example of those observations is shown in Figure 1, in which a similar observation of fertilized eggs is presented for comparison. From this figure, it is clear that the hyaline layer of the

activated egg becomes the thickest at a stage when the monaster is fully formed, which corresponds to the cleaving stage in fertilized eggs. The rhythmical thickening of the hyaline layer is observable at least until the fourth cleavage cycle.

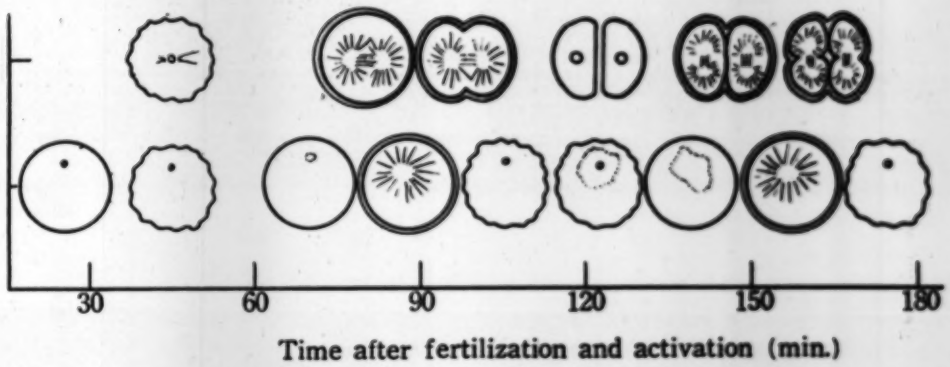


Fig. 1. Cyclic thickening of the hyaline layer in *Clypeaster* eggs (24°C.), fertilized (above) and activated (below).

In the second set of experiments *Temnopleurus* eggs were activated with thymol. Their hyaline layer is much thicker than that of the *Clypeaster* eggs. Figure 2 shows their cyclic changes after activation. The outer surface of the hyaline layer first becomes uneven and then the outline of the egg proper becomes angular soon after the disappearance of the monaster and the reappearance of the nuclear membrane. The hyaline layer which has increased in thickness gradually resumes its original thickness and the egg again becomes spherical. These changes are repeated, synchronizing with the cleavage-activity in normally fertilized eggs.

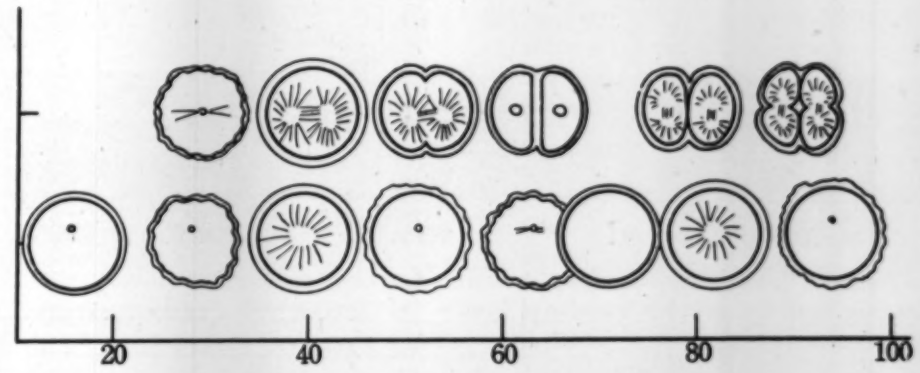


Fig. 2. Cyclic thickening of the hyaline layer in *Temnopleurus* eggs (24°C.), fertilized (above) and activated (below). The fertilization membrane is omitted.

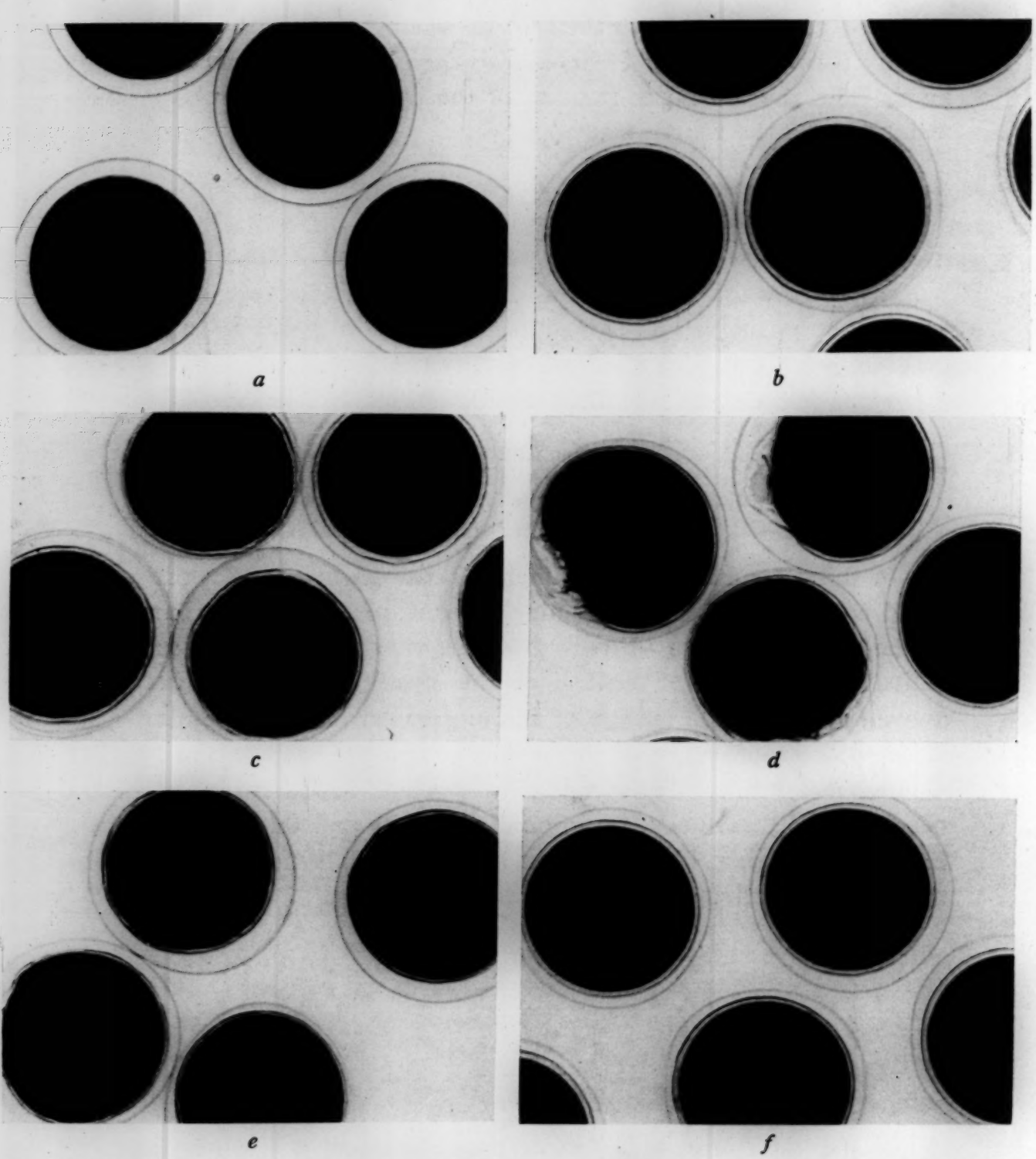


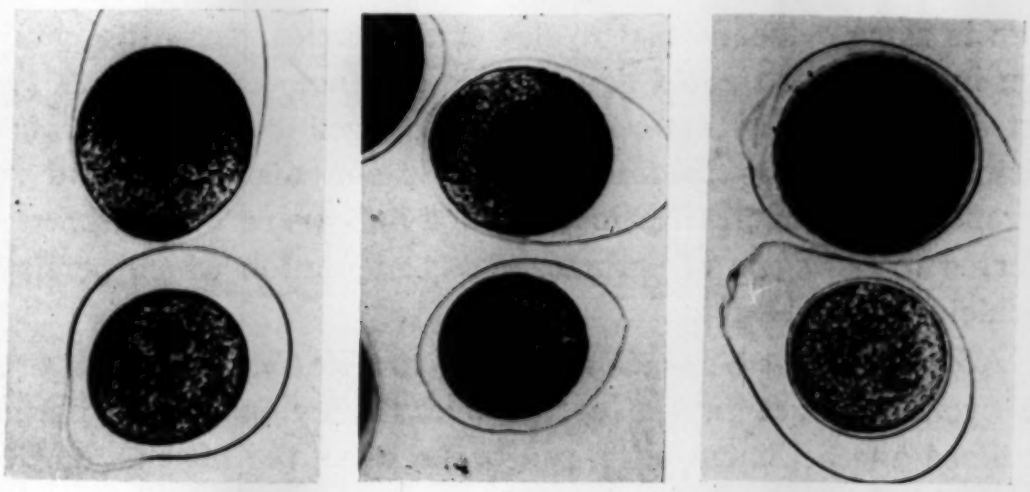
Fig. 3. Cyclic thickening of the hyaline layer in activated *Pseudocentrotus* eggs (18°C.). *a*: 15 minutes after activation. —— *b*: 120 minutes after activation. The hyaline layer becomes very thick.—— *c*: 130 minutes after activation. The outer surface of the hyaline layer becomes uneven.—— *d*: 140 minutes after activation. The outline of the egg proper becomes uneven.—— *e*: 170 minutes after activation. Both the hyaline layer and the egg surface return to their original form. —— *f*: 190 minutes after activation. The hyaline layer becomes thicker again.

Similar results were obtained in *Pseudocentrotus* and *Hemicentrotus* eggs, though their nuclear changes were not so clearly observable as in the *Clypeaster* and *Temnopleurus* eggs. One of the results is shown in Figure 3.

2. Activation of non-nucleated fragments

In the next group of experiments the change in the thickness of the hyaline layer in activated centrifugal egg-fragments, which lack the nucleus, was studied in comparison with centripetal egg-fragments in order to determine whether the cyclic thickening of the hyaline layer takes place without the nucleus.

An example of the experiments with *Hemicentrotus* eggs, is shown in Figure 4. It will be seen from these photographs that even in the non-nucleated centrifugal egg-fragments, a thickening of the hyaline layer appears after activation, synchronizing with a thickening of the hyaline layer in the nucleated centripetal egg-fragments. This seems to suggest that the above-described cyclic change of the hyaline layer is not directly controlled by the nuclear activities. In addition, it should not be overlooked that even activated egg-fragments have the same cleavage cycle as that shown in normally fertilized eggs.



a *b* *c*

Fig. 4. Changes in the thickness of the hyaline layer in both centripetal (above) and centrifugal (below) egg-fragments of *Hemicentrotus pulcherrimus* (20°C.).

a: 10 minutes after activation. —— *b*: 40 minutes after activation. ——*c*: 80 minutes after activation. The hyaline layer becomes thicker in both egg-fragments.

3. Treatment with colchicine

A series of experiments were performed to investigate the effect of colchicine on the cyclic thickening of the hyaline layer. *Hemicentrotus* eggs and egg-fragments divided by centrifuging were activated with butyric acid and 5 minutes later they were placed in a colchicine sea water solution (8×10^{-4} M). In all cases, including those of the non-nucleated egg-fragments, the hyaline layer became the thickest at a stage roughly corresponding to that of the cleavage in normally fertilized eggs.

Discussion

As already mentioned, Monroy and Montalenti (1947), found that the mode of plasmolysis of the egg and the birefringence of the cortical layer cyclically change in the colchicine-treated fertilized egg, in which the aster formation is suppressed. According to Swann and Mitchison (1953), on the other hand, the rigidity and light-scattering properties of the cortex in the fertilized egg change rhythmically even when they are treated with colchicine.

These results suggest that such cyclic changes are independent of the aster formation. But it might not necessarily be concluded that the changes are independent of the nuclear activities, since there is some evidence that cycles of the nuclear activities continue even in colchicine-treated eggs. For instance, Zeuthen (1951) reported that the rhythmic change of oxygen consumption of colchicine-treated eggs is associated with the dissolution and reappearance of the nuclear membrane, and Swann and Mitchison (1953) referred to the cycle of chromosome condensation which persists in colchicine-treated eggs.

Accordingly, it becomes necessary to determine whether or not the cyclic changes in the cortex and the cytoplasm of normally fertilized and activated eggs, can occur when the nucleus has been removed. To answer this question, the author carried out a series of experiments of artificial activation using non-nucleated egg- fragments, and found that the cyclic changes occur without the nucleus. Thus, it may be concluded that at least the rhythmic changes, such as the thickening of the hyaline layer, are not directly controlled by the nucleus.

In addition, it should be noted that the cyclic change of the hyaline layer is not affected by the colchicine treatment, both in activated whole eggs and in activated non-nucleated egg-fragments. This fact is in harmony with the results obtained by the previous workers.

Summary

1. In the activated sea urchin egg the cyclic change in the thickness of the hyaline layer was observed at a stage which corresponds to the cleaving stage in a normally fertilized egg.

2. Even activated centrifugal egg-fragments, lacking the nucleus, showed the rhythmic thickening of the hyaline layer, which synchronized with the cleavage cycles of the fertilized egg.

3. The cyclic change of the hyaline layer was found in activated eggs and egg-halves, even when they were treated with colchicine.

4. From these results, it may be concluded that the cyclic thickening of the hyaline layer is independent of the cycles of the aster formation and nuclear activities.

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ENDODERMAL TISSUES DEVELOPED FROM THE ISOLATED NEWT ECTODERM UNDER THE INFLUENCE OF GUINEA PIG BONE MARROW

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INTRODUCTION

It has been established that the presumptive ectoderm of *Triturus*-gastrulae can be induced to form almost all principal tissues belonging to the ectoderm and mesoderm under the influence of differentiated tissues of various vertebrates. When the induced structures are classified according to regionality, a relatively specific relationship is obvious between the kind of inducing tissues and the regional types of the induced structures. In this respect, the bone marrow of the guinea pig has attracted special attention because of its almost exclusively mesodermal effect, which was first demonstrated by TOIVONEN (1953, 1954). Participation of the presumptive mesoderm in the formation of those mesodermal structures was excluded by TOIVONEN by employing the sandwich technique in which the isolated presumptive ectoderm alone was cultured with a piece of the bone marrow, and still produced abundant mesodermal tissues. It was pointed out that the mesodermal structures induced in the experiments with bone marrow exhibit, in typical cases, the characters of the trunk regions, and are distinguishable from the mesodermal structures induced for instance with guinea pig kidney, which exhibit caudal properties (YAMADA, 1958 a, c, 1959). Further studies revealed that in the ectodermal explant developed under the influence of bone marrow, groups of yolk-rich cells are often included among the mesodermal tissues, and that if the culture is long enough they may express histological characters of endodermal tissues (YAMADA, 1958 b). The present study is designed to test the suggested induc-

[1] Supported partly by the Scientific Research Expenditure and a grant from the Rockefeller Foundation.

tion of the endodermal structures from the presumptive ectoderm by ethanol-treated guinea pig bone marrow. For the purpose, a culture of long duration which allows complete disappearance of yolk platelets in those originally yolk-rich cells was attempted. According to our previous studies (C. TAKATA, in press) of *in vitro* differentiation of the endoderm isolate, only after almost complete utilization of yolk platelets a reliable identification of individual endodermal tissues is possible.

MATERIAL AND METHODS

As the medium for surgical operation and cultivation HOLTFRETER's solution buffered with Tris at pH 7.3–7.4, without antibiotics, but prepared aseptically was exclusively used. Prior to the operation, the bone marrow tissue collected from the frozen bones of guinea pigs was kept in 90% or 70% ethanol at −20°C. for three to 10 days. A small piece of thus treated tissue was washed in Holtfreter's solution, and inserted between two pieces of the presumptive ectoderm isolated from the early gastrula of *Triturus pyrrhogaster*. The average diameter of the isolated ectoderm was about 1 mm., and that of the implant about 0.4 mm.

Each explant thus obtained was placed in a STENDER dish containing *ca.* 30 cc. of the sterile culture medium and covered with the lid. The cultivation was carried out at 18°C., and continued as long as possible, *i.e.* for 30 to 59 days. 30 to 40 days were found necessary for the explant to attain the stage of development, in which the bulk of yolk platelets had disappeared in its endodermal component. Throughout the culture the medium was not renewed. In all phases of the experiment aseptic conditions were maintained. Explants were studied microscopically in sections after fixing with BOUIN's mixture and staining with MAYER's hemalum and picroblauschwarz.

RESULTS

In the present experiment only the explants which could be cultured long enough to allow an analysis of the endodemal differentiation were examined. Most of 92 available explants formed expanded vesicles with melanophores and xanthophores visible from the outside. In 22 of those explants, tail-like protrusions or limb-

like processes grew out from the surface. Microscopic examination confirmed the previous report (YAMADA, 1958, b, 1959) that not only mesodermal structures but also endodermal structures can be induced from the presumptive ectoderm with the bone marrow. Of 106 endodermal structures recorded in all, 97 were classified into pharynx, oesophagus, lung, stomach, or intestine according to the criteria proposed by C. TAKATA (in press). The remaining 9 structures could not be identified as any of those or other tissues, although their endodermal nature was beyond question.

Table 1. Structures induced in the isolated ectoderm by the bone marrow

No. of available explants:	92
No. of explants with any induction:	92(100%)
No. of explants with endodermal induction:	63(68%)
No. of explants containing induction types:	
Pharynx	32(35%)
Branchial pouches	18(20%)
Oesophagus	15(16%)
Lung	5(5%)
Stomach	10(11%)
Intestine	17(18%)
Unidentifiable endodermal tissues	9(10%)
Trunk-notochord	89(97%)
Trunk-somites	64(70%)
Nephric tubules	63(68%)
Limb-rudiment	25(27%)
Heart	6(7%)
Cartilage	15(16%)
Mesenchyme	77(84%)
Mesothelium	53(58%)
Blood cells	31(34%)
Tail-notochord	10(11%)
Tail-somites	4(4%)
Neural tissues	60(65%)
Melanophores	66(72%)
Xanthophores	57(62%)

Pharynx (Fig. 1, 2). The tissue was found as vesicles of various sizes composed of a double layer of squamous non-ciliated cells. In 18 out of 32 cases, the pharyngeal vesicles were accompanied by diverticula resembling the branchial pouches. The pharyngeal vesicles were either inclosed in a thin layer of mesothelium or imbedded

within mesenchyme. The presence of the notochord in the vicinity was very often noticed. Infrequently the pharynx formed a complex with oesophagus, stomach, and intestine.

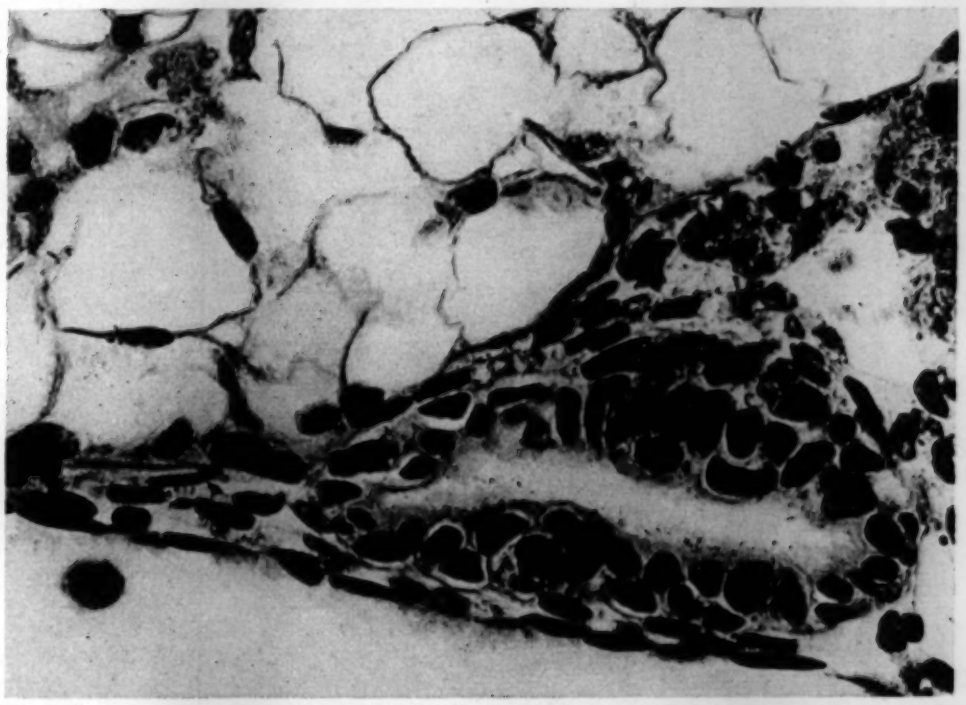

Fig. 1. A pharyngeal vesicle formed in mesenchyme and mesothelium, in contact with a notochord (left, above). All the tissues have been induced by bone marrow in the isolated ectoderm cultured for 50 days. ×360.

Oesophagus (Fig. 2). Vesicles or tubes composed of the one-layered columnar epithelium which was often ciliated were identified as the oesophagus. Those oesophageal vesicles found in 15 explants were either inclosed in mesenchyme or covered by a thin layer of mesothelium. In a few cases the oesophagus formed a common vesicles with pharynx, stomach, or lung.

Lung Rudiment (Fig. 3). The small compact cell mass having large, dark-staining nuclei of the spheroidal or conical shape, and very thin layers of cytoplasm was identified as the lung rudiment. The tissue was found either inclosed in mesenchyme or projected into the "coelomic cavity" of the explant.

Stomach. The gastric vesicles were characterized by the presence of attached structures reminiscent of the gastric glands, and by a

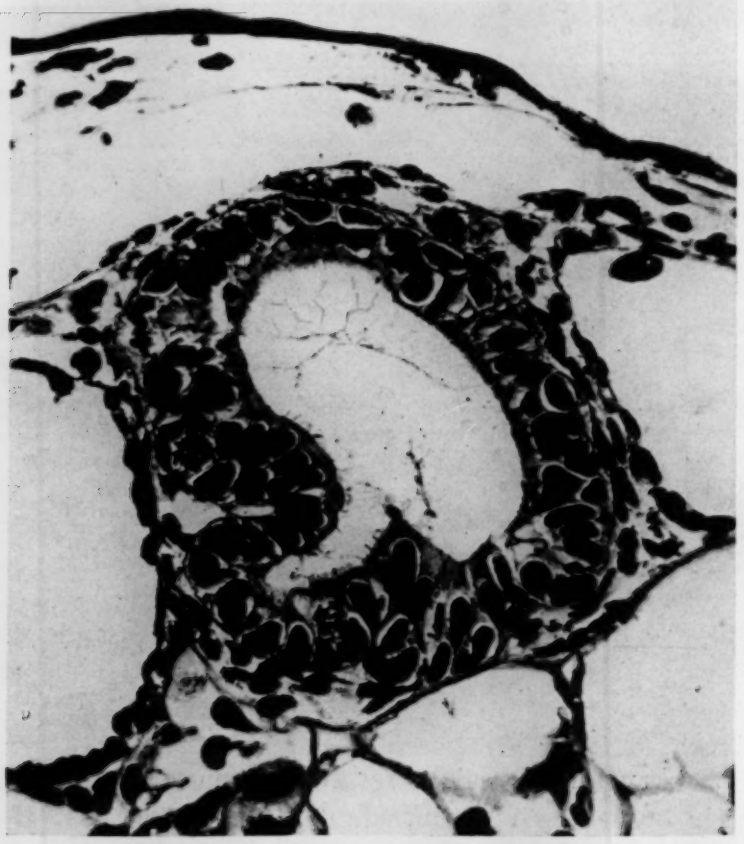

Fig. 2. A vesicle composed of pharyngeal (right, above) and oesophageal epithelia (left, below, the constricted area) differentiated in an ectodermal isolate cultured with bone marrow for 46 days. Note the ciliated cells and mitotic figures in the oesophageal part. The pharyngeal part depicted indicates the condition of the transitional zone toward the oesophagus. ×240.

very uneven internal surface. The one-layered columnar epithelium of the vesicle was composed of cells which were ciliated or non-ciliated, and contained elongated nuclei in their basal part. A mesothelial layer always covered the gastric vesicles. Infrequently a thin layer of muscle cells were observable underneath the mesothelial sheath of the gastric vesicle. In 3 out of 10 cases the stomach tissue formed a common vesicle together with pharynx, oesophagus, or lung.

Intestine (Fig. 4, 5). The intestinal vesicles were composed of

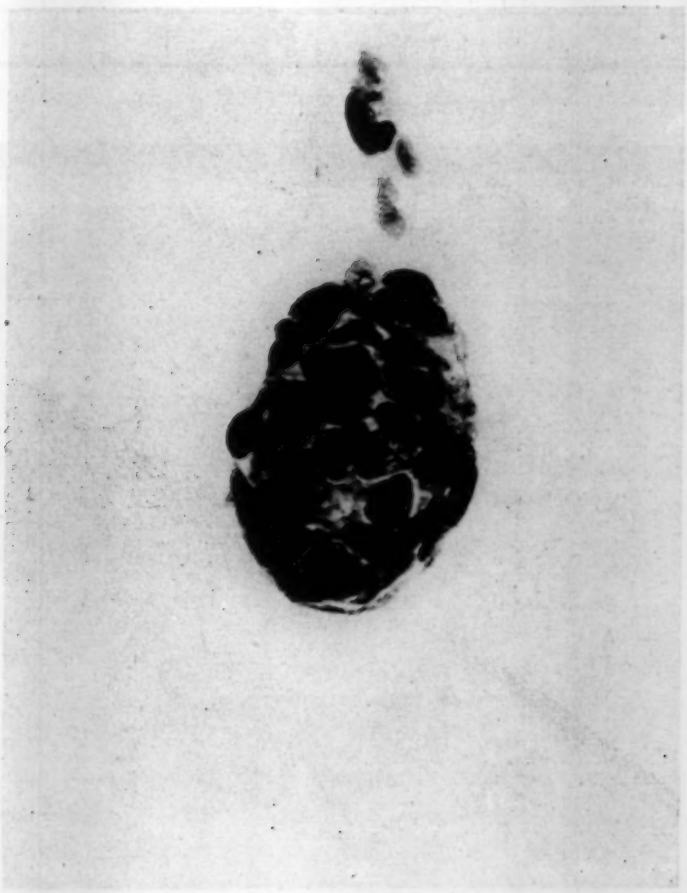

Fig. 3. A compact endodermal tissue resembling the lung rudiment formed in the cavity of an explant cultured for 54 days. ×280.

the one-layered columnar epithelium furnished with the cuticular border and ovoid nuclei located basally. Scattered within the columnar epithelium the goblet cells were frequently found. In 15 cases the intestinal tissue was surrounded by the mesothelium or mesenchyme, and suspended in the "coelomic cavity" formed within the explant. In the remaining two cases they were found imbedded in a mass of undifferentiated mesodermal cells.

As indicated in Table 1, beside the endodermal tissues mesodermal, mesectodermal, and neural tissues were differentiated in the explants. The bulk of the explant was occupied by the mesodermal tissues. Most frequently formed were the trunk-mesodermal structures such as trunk-notochord, trunk-somites, nephric tubules, blood cells (Fig. 6), limb rudiments, mesenchyme and mesothelium. Of some interest was the presence of the heart in the list of induced structures. The heart has not been mentioned as the product of induction by

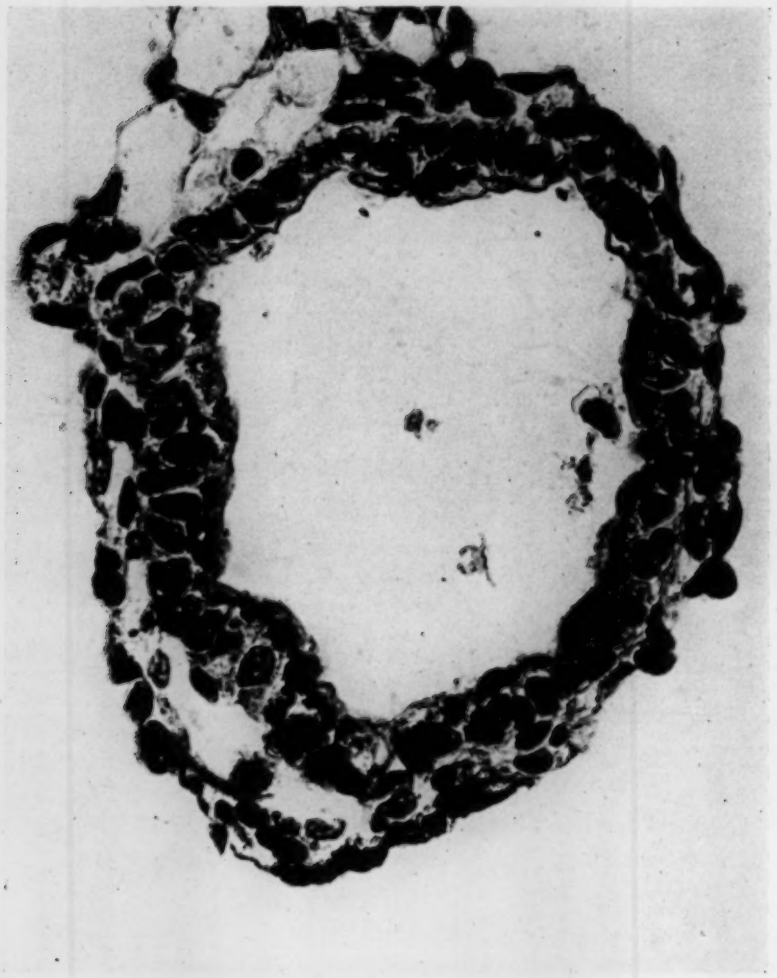

Fig. 4. An intestinal vesicle covered by layers of mesodermal tissues and suspended in a cavity. The explant consisting of the presumptive ectoderm and bone marrow has been cultured for 59 days. ×280.

the bone marrow in the previously reported experiments. In the present study the induction of the heart was noticed for the first time by its rythmical pulsation in the living explants, and later confirmed by histological examination. Also cartilage which was obtained frequently in our experiments has not been reported as yet in similar induction experiments with bone marrow. It is probable that some of the cartilage observed in the explants were of mesectodermal nature, while the other cartilage was of mesodermal nature, the former being associated with the induced pharynx, and the latter with the induced limb rudiment. Thus it may be safely asserted that formation of almost all principal mesodermal structures which are recognizable in the explants of the corresponding deve-

Fig. 5. An intestinal tube formed in an ectodermal isolate cultured with bone marrow for 50 days. Note the cuticular border of the epithelium. ×360.

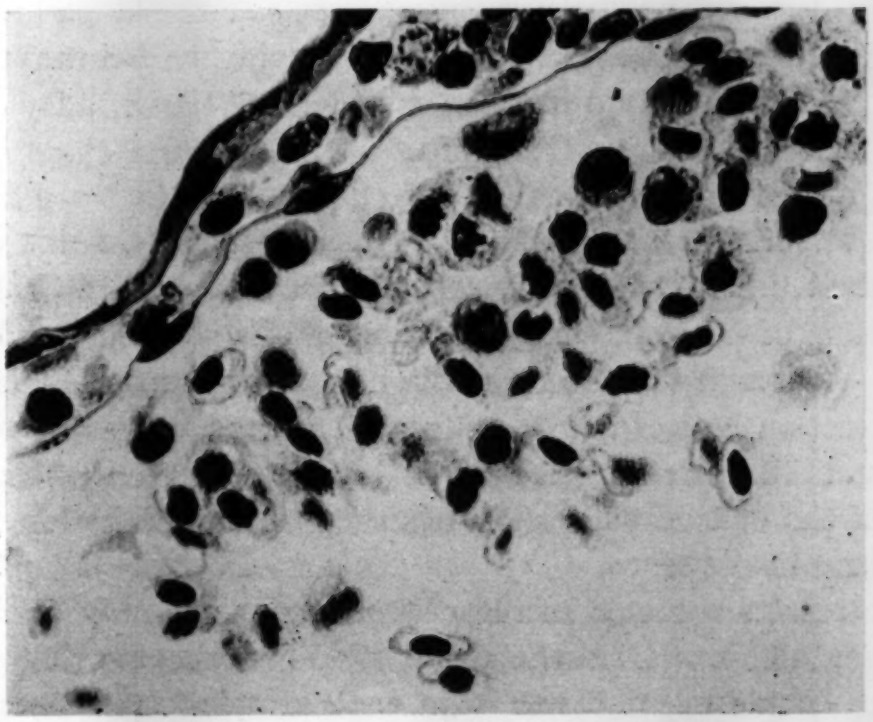

Fig. 6. Various types of blood cells formed from the presumptive ectoderm cultured with bone marrow for 51 days. ×360.

lopmental stage may be evoked in the presumptive ectoderm of the *Triturus* embryo with guinea pig bone marrow.

Discussion

Although a large body of evidence accumulated by experimental embryologists has definitely established that the presumptive ectoderm of the early gastrula of Urodeles can be induced to form mesodermal structures by grafting into mesodermal areas of the embryo, it has not been generally accepted that the endodermal differentiation can be elicited in the same cell-material. However, several papers have suggested the possibility of transformation of the presumptive ectoderm into the endodermal tissues: In an experiment of Mangold (1923) a part of the presumptive ectoderm of the early gastrula of *Triturus cristatus* grafted into the interior of the gastrula of *Triturus alpestris* happened to come in the endodermal area, and assumed the appearance of endodermal cells, fitting harmoniously in the surrounding endoderm. Also among the extensive grafting experiments of Holtfreter (1933) a similar case was reported, in which a xenoplastic combination of *Amblystoma mexicanum* and *Triturus alpestris* was utilized. Since in both experiments no histogenesis of endodermal tissues had occurred at the stage of fixation, their results cannot be taken as decisive evidence for the ability of the graft to differentiate into the endodermal tissue. By grafting the ectoderm of the gastrula of *Amblystoma mexicanum* into the neurula of the same species, Raven (1935) found that a part of the graft differentiated into intestine or liver tissues. In this case the endodermal tissues were in a well advanced stage of differentiation at the time of fixation, enabling the diagnosis of various endodermal tissues. Although the transplantation was homoioplastic, and hence the distinction between host cells and grafted cells was done by circumstantial evidence, little doubt was left as to the ectodermal origin of the endodermal tissue fragments observed beside the corresponding host tissues.

The results reported in the present paper not only confirm the suggestion obtained from the above cited experiments that the presumptive ectoderm is able to form endodermal tissues under given conditions, they also establish that various endodermal tissues are

induced in the isolated presumptive ectoderm by guinea pig bone marrow. However, the ability for endodermal induction is probably not monopolized by the guinea pig bone marrow. For instance an oral communication from the laboratory of Dr. I. KAWAKAMI makes it probable that the lung of the guinea pig also may evoke endodermal differentiation together with the trunk-mesodermal differentiation in the isolated ectoderm of the *Triturus* gastrula.

The situation that in the ectodermal explant containing bone marrow a large amount of mesodermal structures, and a smaller amount of endodermal structures are formed leads us to the question whether the endodermal differentiation may or may not be due to a secondary inductive effect caused by the primarily induced mesoderm on the remaining ectoderm. Although the possibility cannot be wholly excluded, one strong argument against it is the fact that hitherto no clear case is known in which endodermal tissues are induced in the ectoderm by the mesoderm in spite of the extensive literature pertinent to the point.

On the other hand, it appears probable that the endoderm once formed is dependent for its further development upon the mesodermal tissues coexisting in the explant, since it has been established that differentiation of endodermal tissues out of the yolk-rich endodermal cell-groups is only possible in the presence of the mesoderm or mesectoderm and that the regional nature of the endodermal tissue is controlled to some extent by the type of mesodermal tissues present in its environment (T. S. OKADA, 1957; C. TAKATA, in press).

In Table 2 frequencies of individual mesodermal structures found in the explants containing endodermal structures are compared with those of the explants containing no endodermal structures. Significant differences between two groups are found in the frequencies of blood cells, mesothelium, and mesenchyme. The results may mean that the formation of endodermal tissues out of the ectoderm tends to occur under the condition favourable for the formation of those mesodermal tissues.

In the normal development of the endodermal tissues the tissue specificity appears to depend firstly on the intrinsic tendency of the endodermal areas and secondly on the nature of the mesoderm with which it comes into contact. If we assume that a similar situation holds

Table 2. Types of mesodermal induction in the explants with and without endodermal induction

Total number of available explants:	92	
Number of explants:	With endodermal induction 63	Without endodermal induction 29
Number of explants containing induction types:		
Trunk-notochord	58(92%)	21(72%)
Trunk-somites	41(65%)	23(79%)
Nephric tubules	46(73%)	17(59%)
Limb-rudiment	19(30%)	6(21%)
Blood cells	29(46%)	2(7%)
Mesenchyme	60(95%)	17(59%)
Mesothelium	47(75%)	6(21%)
Melanophores	50(79%)	16(55%)
Xanthophores	44(70%)	13(45%)
Cartilage	11(17%)	4(14%)

in the case of the endoderm formed in the ectodermal explants under the influence of bone marrow, it might be expected that the quality of endodermal structures is *partly* decided by the nature of the mesodermal tissue with which they come into contact. With this possibility in mind Table 3 is constructed. Three representative endodermal tissues are selected, and their relations with various mesodermal structures are studied. Although no definite difference is observable in the frequencies of the majority of the mesodermal structures between three groups of explants, significant differences are detected in the frequencies of trunk somites and blood cells between the explant-group carrying the pharynx and that carrying

Table 3. Relationship of individual mesodermal structures to the representative endodermal structures

Total number of available explants:	92		
Number of explants containing:	Pharynx 32	Stomach 10	Intestine 17
Number of explants containing:			
Trunk-notochord	31(97%)	8(80%)	15(88%)
Trunk-somites	22(69%)	6(60%)	6(35%)
Nephric tubules	21(66%)	8(80%)	13(76%)
Limb-rudiment	7(22%)	3(30%)	6(35%)
Blood cells	11(34%)	4(40%)	12(71%)
Mesenchyme	31(97%)	10(100%)	16(94%)
Mesothelium	27(84%)	9(90%)	13(76%)

the intestine: The pharynx is formed more frequently in the explant possessing somites, and less frequently in the explant possessing blood cells, whereas the intestine is formed more frequently in association with blood cells, and less frequently with somites. It should be further added that in the explants carrying the intestine the average size of the notochord appeared much smaller than that found in the explant carrying the pharynx. All the results do not contradict the notion developed in the study of the morphogenetic effects of mesodermal structures on the isolated endoderm that differentiation of the pharynx is favoured in the presence of the dorsal trunk mesoderm, and that of the intestine is favoured in the presence of the ventral trunk mesoderm (C. TAKATA, in press).

Although as yet no study has been undertaken concerning the events occurring in the ectodermal cells during its transformation into the endodermal cells, inhibition of cell division in the early phase appears to be one of the events. This is suggested by the observation that the endodermal cells found in the explant cultured with bone marrow for *ca.* 10–14 days are approximately of the same size as the cells of the presumptive ectoderm at the beginning of the culture, while other cells of the explant are definitely much smaller than those endodermal cells. At the stage of development, the size of the cells derived from the same embryonic area of the uniform cell size depends primarily on the rate of cell-division.

Experiments conducted in our laboratory has revealed that the ability of inducing endodermal structures from the presumptive ectoderm is intimately associated with the ability of inducing mesodermal structures, and that both abilities are carried by a protein of the supernatant fraction of the bone marrow, which is precipitable at pH 4.7, and sedimentable with 50% saturation of ammonium sulphate (cf. YAMADA, 1958 b). That also in the heat lability both abilities are not dissociable has been suggested by the heating experiment reported earlier (YAMADA, 1958 a, 1959).

SUMMARY

The presumptive ectoderm of the early gastrula of *Triturus pyrrhogaster* was isolated and cultured with a piece of ethanol-treated bone marrow of the guinea pig. The explant was allowed to develop

long enough (30–59 days at 18°C.) so that the endodermal tissues induced by bone marrow revealed their histological characteristics. Pharynx, oesophagus, lung, intestine, and stomach were identified among the endodermal tissues which were induced together with the various mesodermal tissues. The possible influence of the mesodermal tissues induced together in the explant on the differentiation of the endodermal tissues is discussed.

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DATA ON THE SPAWNING OF *COMANTHUS JAPONICA* BETWEEN 1937 AND 1955*

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INTRODUCTION

Lunar periodicity in the reproductive activity of various animals has repeatedly been pointed out in the literature (OKUDA, 1939; CASPERS, 1951). One of the authors of the present paper has reported observations made from 1937 to 1940 indicating that the spawning habit of the crinoid, *Comanthus japonica* is related to the phase of the moon (DAN, K. and J. C. DAN, 1941). Although these observations have been extended to the present, this article will concern chiefly the data covering the period from 1937 to 1955, in order to emphasize a new primary factor influencing spawning which analysis of these data reveals.

This research has been conducted at the Misaki Marine Biological Station extending over twenty years. The authors express their deep indebtedness to the directors and staff members of the Station.

DESCRIPTION OF THE MATERIAL

Comanthus japonica is a non-stalked crinoid common in the vicinity of Misaki. It has 40 arms surrounding the central disc and each arm is provided with many pinnules arranged alternately on the two sides. The general body color is blackish brown, although in many individuals the tip of each pinnule has a small color patch which varies from lemon yellow to reddish orange according to the individual.

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* This series of observations was supported in part by the Research Expenditure of the Ministry of Education given to the senior author from time to time.

In the breeding season, the pinnules along roughly two-thirds of the arms from the base develop gonads within them (genital pinnules). As a result, the sexes can be identified by examining the contents of the pinnules.

Spawning of the Eggs

According to the records of spawning dates between 1937 and 1940 cited above (Dan, K. and J. C. Dan, 1941), spawning occurred once a year sometime in the early or middle part of October, the dates coinciding with or falling very close to either the first or the third quarter of the moon Moreover, the period of spawning on the spawning day began almost exactly at 3 o'clock in the afternoon and ended within an hour or so.

During about one month preceding spawning, the oogonia begin to grow in size. In the last week or 10 days, the oocyte surface undergoes a series of morphological changes which continue until spawning (see Dan, J. C. and K. Dan, 1941). Around noon of the spawning day, the oocytes are liberated from the chorions which have been investing them and go through the reduction divisions lying free in the ovarian lumen (Dan, K., 1952). Finally at about 3 o'clock, the mature ova are spawned by the rupture of the pinnule walls.

Relation between Spawning and Moon Phase

Spawning dates together with other information which may have a close bearing on the spawning phenomenon are presented in Table 1. But some comments about these data may not be superfluous.

Among the spawning dates which are given in the second row of the table, data for 1941, 1944 and 1954 are missing. This is due to the fact that in 1941 the eggs failed to mature sufficiently, and in 1944, although oogenesis reached a somewhat more advanced stage, the eggs were not spawned and eventually disintegrated within the pinnules. In short, in these two years spawning did not take place. In 1952, although a major spawning took place on Oct. 10th, a fair number of animals were seen spawning on the following day as well. In 1954, the degree of maturation was not uniform among the population and a spawning day as such was not found. However, on Oct.

15th, one female kept in a live cage from the previous day and brought to the laboratory at 2 o'clock that afternoon was found to have spawned to a slight degree at 4.30. But judging from the fact that a survey of the habitat made at about 4 o'clock of the 17th revealed many unspawned females, the authors believe that the vast majority of females failed to spawn and the sexual products must have degenerated within the ovaries.

The 3rd row of the table gives the dates of both the first and third quarter moons occurring close to the spawning dates. The most perplexing feature of these data is the fact that the spawning occurs in some years in connection with the first quarter moon and in other years with the third quarter moon. This fact will be considered in more detail below.

The 5th row of the table indicates the displacement of the spawning dates from their respective nearest quarter moons in absolute numbers of days (ave. 2.06 days).

The last row gives the number of synodic months from the nearest quarter moon of one year to that of the next year, taking one synodic month as 29.52 days. It will be noticed that 12 and 12.5 synodic months alternate with some regularity.

Figure 1 illustrates the correlation between the spawning dates (ordinates) and those of quarter moons (abscissae) in a graphic

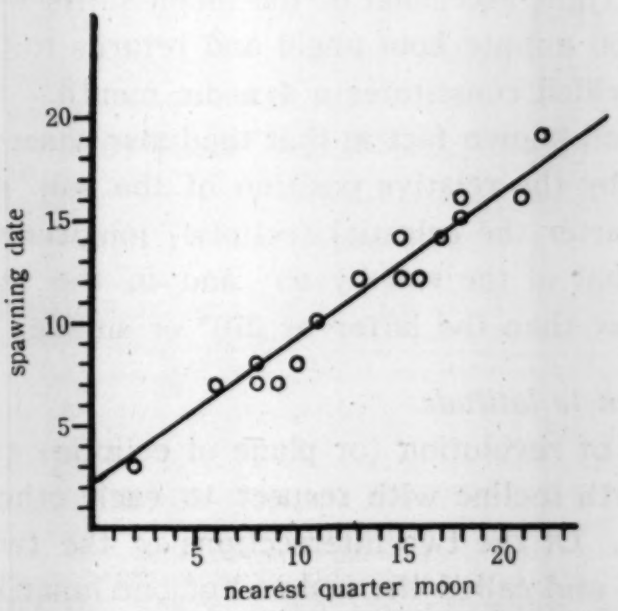

Fig. 1

form. The straight line is the line of regression which is represented by the formula

$$Y = 0.70\,X + 2.33$$

PEARSON's correlation coefficient, r, turns out to be extremely high, i.e.,

$$r = 0.971$$

SPAWNING DATES AND RELATIVE POSITIONS OF THE MOON TO THE EARTH

In the preceding section, a very high correlation has been shown to exist between spawning and the quarter moons. Therefore, the next step is to examine the relative position of the moon in reference to the earth and to analyse its relation to spawning. If the amount of the moon's reflection or the degree of polarization of the reflected light were a determining factor for spawning, as has been proposed in the past (CLARK, L. B. and W. N. HESS, 1940; SEMMENS, E. S. 1932), it will depend upon the angle of incidence from the sun to the moon and also to that from the moon to the earth.

(*a*) *Relation to longitude*

It is an every-day experience that the rise of the moon at any one place shows a daily delay of about 50 minutes. Expressing this otherwise, the right ascension of the moon shifts from the west to the east by a 50 minute hour-angle and returns to the original point in 29.52 days which constitutes a synodic month.

Another well-known fact is that the lunar phase (wax and wane) is determined by the relative position of the sun, moon and earth. In the first quarter the celestial (ecliptic) longitude of the moon is greater than that of the sun by 90° and in the third quarter the former is larger than the latter by 270° or smaller by 90°.

(*b*) *Relation to latitude*

The plane of revolution (or plane of ecliptic) of the moon and that of the earth incline with respect to each other by 5°8′ 43″ 43 on an average. Of the two intersections of the two, one is taken as a reference and called the node. For one lunar circuit, from one ascending traverse of the equator to the next of the same direction,

Year (*leap	0	'51	'52*	'53	'54	'55	mean
Spawning date	5	7	10	12	—	7	
Dates of quarter mo in the latter half of S (IX), Oct. and first of Nov. (XI). ( ): nearest quarter m to spawning			27/IX			24/IX	
		23/IX	(11)	30/IX	17/IX	(9)	
	3)	(8)	27	(16)	5	24	
		23			19		
Discrepancy between and the nearest qua	3	+1	+1	+4		−2	+1.56
Difference in absolute		1	1	4		2	2.06
Synodic months betwe moons of adjacent y		12	12.5	12.5	—	—	

Year (*leap	0	'51	'52*	'53	'54	'55	mean
Spawning date		7	10	12		7	
Dates of southernmos most positions of th (IX) and Oct. ( ): norther		7	(9)	13	(19/IX) 3 (16)	(7)	
Declination of southe northernmost positi +: north, −:	6°	−28.4°	+27.5°	−26.3°	±24.7°	+23.0°	
Discrepancy between and that of expected moon		0	−1	+1		0	+0.19
Difference in absolute		0	1	1		0	1.31

approximation that the years when the disparity was large were concurrently the years when the declination was smaller than 22°

Year (*leap year)		'37	'38	'39	'40*	'41	'42	
Spawning dates (Oct.)		12	3	7	8	—	14	
Dates of quarter moons in the latter half of Sept. (IX), Oct. and first half of Nov. (XI). ( ): nearest quarter moon to spawning	1st quart.			21/IX		26/IX		
	3rd quart.	27/IX	17/IX	(6)	25/IX	14	3	21
	1st quart.	(13)	(2)	20	(8)	28	(17)	
	3rd quart.	27	17		23		2/XI	(
Discrepancy between spawning date and the nearest quarter moon		+1	−1	−1	0		+3	
Difference in absolute magnitude		1	1	1	0		3	
Synodic months between quarter moons of adjacent years		12	12.5	12.5	—	—	12.5	

Year (*leap year)	'37	'38	'39	'40*	'41	'42	
Spawning dates (Oct.)	12	3	7	8		14	
Dates of southernmost and northernmost positions of the moon in Sept. (IX) and Oct. ( ): northernmost	10	30/IX	(5)	7	28/IX (12) 25	16	(
Declination of southernmost and northernmost positions +: north, −: south	−21.9°	−20.4°	+19.0°	−18.4°	±18.4°	−19.0°	+2
Discrepancy between spawning date and that of expected position of the moon	−2	−3	−2	−1		+2	
Difference in absolute magnitude	2	3	2	1		2	

Table 1

42	'43	'44*	'45	'46	'47	'48*	'49	'50	'51	'52*	'53	'54	'55	mean
4	16	—	12	16	19	8	14	15	7	10	12	—	7	
		26/IX					29/IX			27/IX			24/IX	
3	21/IX	9	30/IX		8	25/IX	(15)	4	23/IX	(11)	30/IX	17/IX	(9)	
7)	7	25	(15)	3	(22)	(10)	29	(18)	(8)	27	(16)	5	24	
XI	(21)			(18)	6/XI	26			23			19		
-3	+5		+3	+2	+3	+2	+1	+3	+1	+1	+4		−2	+1.56
3	5		3	2	3	2	1	3	1	1	4		2	2.06
12.5	—	—	12.5	12.5	12	12.5	12.5	12	12.5	12.5	—	—		

Table 2

12	'43	'44*	'45	'46	'47	'48*	'49	'50	'51	'52*	'53	'54	'55	mean
4	16		12	16	19	8	14	15	7	10	12		7	
6	(20)	26/IX (8) 23	13	(17)	20	9	(14)	16	7	(9)	13	(19/IX) 3 (16)	(7)	
0.0°	+20.3°	±21.8°	−23.6°	+25.4°	−26.9°	−27.8°	+28.5°	−28.6°	−28.4°	+27.5°	−26.3°	±24.7°	+23.0°	
2	+4		+1	+1	+1	+1	0	+1	0	−1	+1		0	+0.19
	4		1	1	1	1	0	1	0	1	1		0	1.31

as a reference and called the node. For one lunar circuit, from one ascending traverse of the equator to the next of the same direction,

i.e., one tropical month, is 27.32 days in average. Therefore, it results that the declination of the moon swings northward and southward with a period of 27.32 days, corresponding to a tropical month. On the other hand, owing to the wobbling of the planes, the node itself rotates on the ecliptic in a period of 18.59(989) years. On the basis of the above two propositions, the maximum angle of the swing fluctuates from ±18°18′ to ±28°36′ in a period of 18.6 years corresponding to a complete circuiting of the node on the ecliptic.

It will also be pointed out for convenience that the number of tropical months and that of synodic months within one solar year coincide in decimal figures while they differ in integers. That is to say,

1 solar year = 365.242186 days = 13.36826 tropical months
= 12.36826 synodic months.

Now the practical outcome of the above considerations is that every year during one month from late September to late October, the new moon and the full moon approximately coincide with the crossing of the equator by the moon, while the first and third quarters occur nearly simultaneously with the southernmost and the northernmost positions respectively. Moreover, this condition recurs year after year since the numbers of tropical and of synodic months included in one solar year coincide in decimal figures.

As the result of this, it now becomes necessary to examine the relation between spawning dates and the southernmost or the northernmost positions of the moon. The data are given in Table 2. In this table two things which differ from Table 1 are that (1) instead of the quarter dates, the dates of the southernmost or the northernmost positions of the moon nearest to the spawning dates are given in the 3rd row and (2) declination (angle of deviation of the moon from the equator) is shown in the next row.

The average of the discrepancy between the dates of spawning and of the expected position of the moon in absolute magnitude turns out to be 1.37 days (6th row). This figure is smaller than the disparity between the spawning dates and those of the quarters shwon in Table 1 (2.06 days). Table 2 further shows in a rough approximation that the years when the disparity was large were concurrently the years when the declination was smaller than 22°

(left half of the table), while in the years when the declination was larger than 22°, the accuracy of the prediction was high and the error remained under 1 day (right half). When the prediction is made by the quartermoon, the situation is almost opposite (see Table 1) so that predictions by the declination and those by the quarter moon supplement each other.

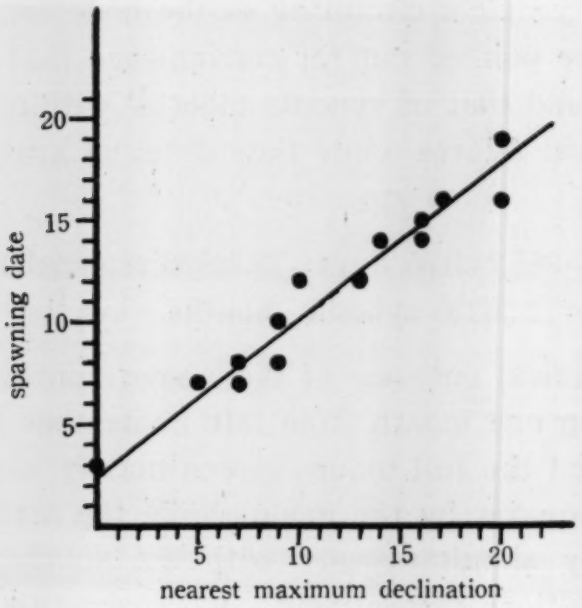

Fig. 2

The dates of spawning and those of either the southernmost or the northernmost position of the moon are plotted in Figure 2. The line of regression is expressed by the formula

$$Y = 0.77\,X + 2.43$$

On calculating PEARSON's correlation coefficient, it is found that

$$r = 0.975$$

This figure is very slightly better than the former correlation coefficient obtained by the quarter moon.

All the data are illustrated in graphic form in Figure 3.

(*c*) *Distance between moon and earth*

The interval required for the moon to pass the point nearest to the earth twice is the anomalistic month; one anomalistic month = 27.55 days. Within this period the distance from the earth fluctuates

about 10%. However, no possible relation could be found between this and the spawning activity of *Comanthus*.

Considerations

(*a*) *Distribution of the spawning days on the calendar*

As is evident from Tables 1 and 2 and Figure 3, the spawning dates of *Comanthus japonica* are mostly concentrated around the first half of October. Within the period treated in this paper, the earliest case took place on Oct. 3 in 1938* and the latest case was that of Oct. 19 in 1947.

Now if it may be assumed that spawning is, for some unknown reason, restricted to this period of 2–3 weeks which will be called the "breeding period", an interesting proposition can be foreseen. Since one synodic month (interval between two new moons or two full moons) which is 29.52 days is divided into four quarters, in the majority of cases this special period for breeding can cover two successive quarter moons and never three. In other words, for one year, there is one first quarter and one third quarter moon which may be correlated with spawning. Furthermore, in some years, the pair of quarter moons is shifted so far to one side that there is only one quarter moon occurring during the breeding period. This was the situation in 1937, 1940, 1941, 1944, 1948 and 1952 (see Figure 3).

Yet there seem to be further differences even among these years, since in 1937, 1940, 1948 and 1952 spawning took place, while it failed in 1941 and 1944. While the moon-phase in both these latter years was the third quarter, the occurrence of a third quarter among the group of four years in which spawning did occur (1952) indicates that the stimulus to spawning cannot be attributed to one moon-phase or the other.

(*b*) *Determining factors in selection of spawning date*

The most striking overall feature of Figure 3 is the fact that the line follow a descending course and after hitting the lower limit (border of the breeding period on the early side), they jump back to a higher level (1940–1941–1942). The second noticeable feature is that the spawning dates are grouped in triplets in which the ar-

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* After 1955, cases were observed in which spawning took place earlier than this period but they do not interfere with the present discussion.

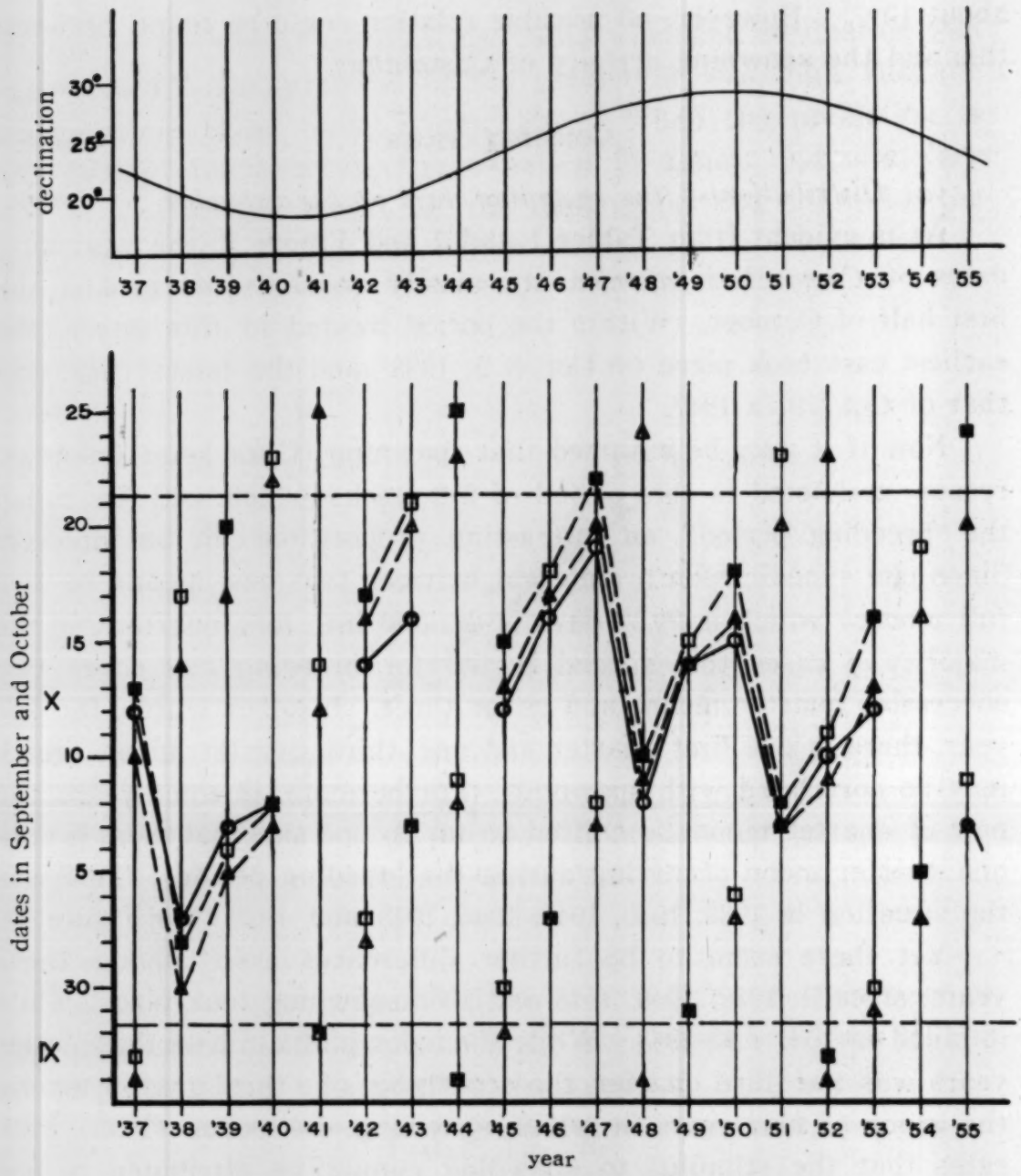


Fig. 3. Relation between spawning and the moons
■: 1st quarter □: 3rd quarter ▲: southernmost
△: northernmost — - —: limit of breeding period

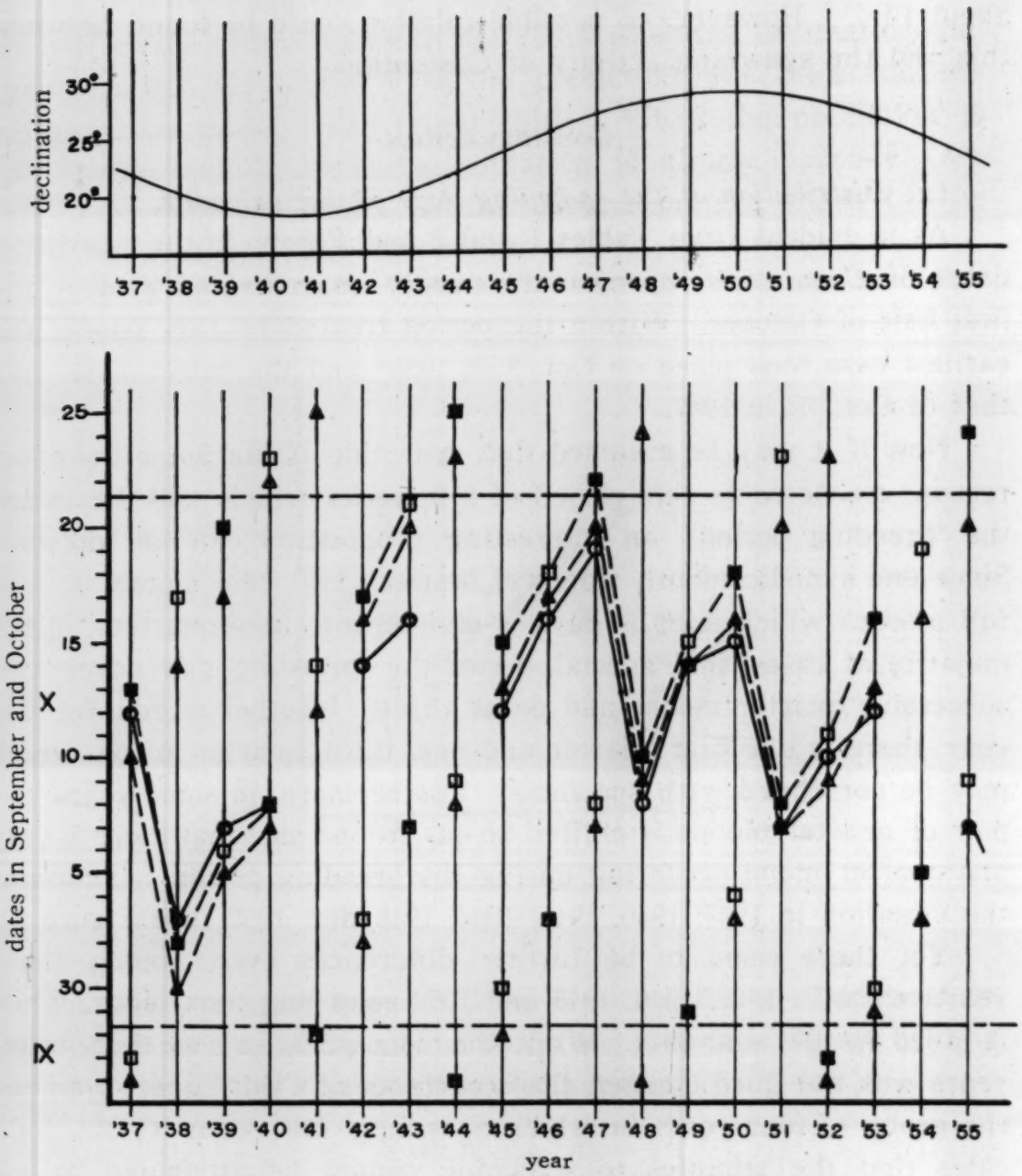
rangement of 1st—3rd—1st quarter moons is consistent.

On the basis of these two tendencies, the observed data can be organized into an almost perfect pattern. The period between 1945 and 1953 will be taken first as an example, since during this period the triplet of 1st—3rd—1st quarter moons was repeated three times. It is also pointed out that from Table 1, we learn that the three

members of a triplet are spaced at intervals of 12.5 synodic months while the interval from one triplet to the next is 12.0 synodic months.

Whatever the reason may be, such a system can be expressed as the method of selecting through successive years the quarter moons which occur on dates as close together as possible on the solar calendar. Let us consider this by concrete cases shown in Figure 3. In 1948, for example, spawning was correlated to the first quarter moon which occurred on Oct. 10. The quarter moon nearest to this date in 1949 was the third quarter of Oct. 15, the first quarter having been 11 days earlier. In 1950 the quarter nearest to Oct. 15 was the first, on Oct. 18, the previous quarter having again been 11 days earlier than the 15th. This situation has the following arithmetical basis. As was stated before, there are 12.37 synodic months in 1 solar year. However, the spawning dates of *Comanthus* can only be shifted by a step of 0.5 synodic months (1st and 3rd quarters only) so that the closest figure to 12.37 which is realizable for *Comanthus* spawning would naturally be 12.5 synodic months with the difference of $0.5-0.37=0.13$ synodic months or $29.52\times0.13=3.84$ days. In order to fulfill this arithmetical condition, the spawning date has to be switched from the first quarter to the third quarter and from the third quarter back to the first quarter again. For convenience, this kind of adjustment will be called half-unit-adjustment.

On the other hand, if the same system is continued for 4 years, a new difficulty arises. By the 4th year, the spawning date will become later than that of the 1st year by $(0.5-0.37)\times3=0.39$ synodic months or $3.84\times3=11.52$ days. In the meantime, the other quarter moon, which was 0.5 unit ahead at the beginning, will be pushed back toward the initial spawning date to the same degree (0.39 synodic months) so that it is separated from the initial date by only $0.5-0.39=0.11$ synodic months or $29.52\times0.11=3.25$ days. Consequently, in the 4th year, the shift in the spawning time can be made smaller by changing back to the earlier quarter. This involves a jump from one set of triplets to the next with the interval of 12.0 synodic months where two 1st quarters occur in seccession (as between 1947–1948, and 1950–1951). This will be referred to as whole-unit-adjustment. The above interpretation, although it may sound



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Whatever the reason may be, such a system can be expressed as the method of selecting through successive years the quarter moons which occur on dates as close together as possible on the solar calendar. Let us consider this by concrete cases shown in Figure 3. In 1948, for example, spawning was correlated to the first quarter moon which occurred on Oct. 10. The quarter moon nearest to this date in 1949 was the third quarter of Oct. 15, the first quarter having been 11 days earlier. In 1950 the quarter nearest to Oct. 15 was the first, on Oct. 18, the previous quarter having again been 11 days earlier than the 15th. This situation has the following arithmetical basis. As was stated before, there are 12.37 synodic months in 1 solar year. However, the spawning dates of *Comanthus* can only be shifted by a step of 0.5 synodic months (1st and 3rd quarters only) so that the closest figure to 12.37 which is realizable for *Comanthus* spawning would naturally be 12.5 synodic months with the difference of $0.5-0.37=0.13$ synodic months or $29.52 \times 0.13 = 3.84$ days. In order to fulfill this arithmetical condition, the spawning date has to be switched from the first quarter to the third quarter and from the third quarter back to the first quarter again. For convenience, this kind of adjustment will be called half-unit-adjustment.

On the other hand, if the same system is continued for 4 years, a new difficulty arises. By the 4th year, the spawning date will become later than that of the 1st year by $(0.5-0.37)\times 3=0.39$ synodic months or $3.84\times 3=11.52$ days. In the meantime, the other quarter moon, which was 0.5 unit ahead at the beginning, will be pushed back toward the initial spawning date to the same degree (0.39 synodic months) so that it is separated from the initial date by only $0.5-0.39=0.11$ synodic months or $29.52\times 0.11=3.25$ days. Consequently, in the 4th year, the shift in the spawning time can be made smaller by changing back to the earlier quarter. This involves a jump from one set of triplets to the next with the interval of 12.0 synodic months where two 1st quarters occur in seccession (as between 1947–1948, and 1950–1951). This will be referred to as whole-unit-adjustment. The above interpretation, although it may sound

somewhat artificial, may not be too teleological, since whenever our predictions failed, the spawning dates were shifted so as to make them converge toward the mid-point of the breeding period. This mid-point seems to be the 7–8th Oct., since in four cases of exact coincidence of prediction and spawning date, three occurred at this time.

The two kinds of adjustment described above are, however, still not adequate to stabilize the spawning date satisfactorily. In fact, with the half-unit- and whole-unit-adjustments working together, they over-compensate the change and gradually push the dates toward the early side, as appears in the general descending trend of the lines of Figure 3. Consequently, the spawning dates are eventually pushed so far forward as to reach the early limit of the breeding period, and a third kind of larger scale adjustment is required to pull back the date again toward a higher level. This, which apparently happened in 1942, will tentatively be called a large-scale-adjustment.

These three conceivable types of "adjustment" have been adopted merely as a convenient method of presenting the steps in the process of analysing the *Comanthus* spawning situation, and not with the idea that any teleological cause is operating to keep the spawning dates within as narrow a range as possible.

(*c*) *Possible factors underlying failure of spawning*

After laying down this much foundation, we are now in a position to consider the causes of the failure to spawn in 1941, 1944 and 1954. 1941 was a year of transition in which a large-scale-adjustment took place. After the completion of a triplet in 1940, a forward jump with an interval of 12.0 synodic months was to be expected. But in this particular case, such an adjustment brings the date out of bounds, to Sept. 28 (Fig. 3). In other words, in 1941 there was only one quarter moon included within the breeding period which, however, happened to be a "wrong" moon. Although there is no way to prove that this was actually the cause, it is at least an attractive possibility to account for the failure. It may be more than a mere coincidence that 1941 was a year in which the declination reached the minimum—18.4°.

In 1942, a new triplet was started; in other words, 1941 does

not belong to any particular triplet, but falls into a gap between two sets of triplets. How can this be explained by the present scheme?

At this point, the reader is asked to recall that the declination of the moon goes through a maximum and a minimum, one cycle of 18.6 years corresponding to a complete circuiting of the node on the ecliptic. An interesting situation, then, is that six of the triplets can be fitted into such a cycle of 18.6 years with a residue of 0.6 years. After two cycles, the residue will add up to 1.2 years. In other words, in every 37 years there would be one odd year which must be disposed of in some way or another. Biologically this method of disposal could take the form of skipping the spawning, and if so, this would likely happen in a year of minimum declination, since the spawning in such a year would be dubious anyway. If such a bold assumption is allowable, it can be predicted that the next occasion of this sort will come in 1978.

The second case of failure was 1944, which is a rather simple case to explain from our proposed system. As is clear from Figure 3, this year corresponds to the last member of a triplet However, the usual half-unit-adjustment will throw the spawning date out of the breeding period, while the other quarter moon remaining within the period is a wrong moon. In other words, no quarter moon was available.

For 1954, it was expected that spawning ought to take place in the vicinity of the 5th of October, and both the facts that the date was well within the spawning period and the declination was larger than 22° should have made this a favorable year. Spawning nevertheless failed to occur, a fact for which the authors have no explanation. In 1955, the rule was borne out. The exception to the general rule encountered in 1954 may not be grave enough to annul the rule; it perhaps indicates that there is another factor involved in determining the spawning date, which is superimposed on the general rule. The authors hope to analyse this factor in future study.

Discussion

In Table 3, examples of correlation between reproductive activity

and the first or third quarter of the moon are collected from compilations of OKUDA (1939) and of CASPERS (1951).

Table 3

Moon	Species	Group	Investigator
1st quarter	*Syllis amica*	Polychaeta	HERPIN, 1926
3rd quarter	*Obelia geniculata*	Hydrozoa	ELMHIRST, 1925
	Eulalia punctifera	Polychaeta	FAGE and LEGENDRE, 1926, 1927
	Odontosyllis enopla		GALLOWAY and WELCH, 1911
	O. sp		CRAWSHAY, 1935
	Nereis succinea		DURCHON, 1948
	N. pelagica		HERPIN, 1925
	N. irrorata		HERPIN, 1925
	N. longissima		GALLIEN, 1936
	Leptonereis glauca		FAGE and LEGENDRE, 1923, 1927
	Eunice viridis		COLLIN, 1892; CORNEY, 1922
	E. harassii		HERPIN, 1926
	Staurocephalus rudolphi		FAGE and LEGENDRE, 1927
	Chaetopleura apiculata	Amphineura	CROZIER, 1922; GRAVES, 1922
	Chiton tuberculatus		CROZIER, 1922
1st or 3rd q.	*Eunice fucata*	Polychaeta	MAYER, 1900, 1902, 1908, 1909
1st and 3rd q.	*Platynereis dumerilli*	Polychaeta	HEMPELMANN, 1911
	Prionospio cirrifera		FAGE and LEGENDRE, 1927
	Pr. malmgreni		FAGE and LEGENDRE, 1927
	Arenicola cristata		OKADA, 1941

Concerning the best-studied example, that of the Pacific Palolo worm *Eunice viridis*, COLLIN's remark (1897) about the spawning intervals of successive years is particularly interesting. He says the interval is usually 12 synodic months with an interval of 13 synodic months interposed once every 3 years. Comparing this pattern of 12—12—13 with that of *Comanthus*, which is 12.5—12.5—12.0, it is immediately apparent that the end results turn out to be the same, the only difference being that, in the Palolo, adjustment takes place entirely by whole units. As the result of this coarse adjustment, the spawning of *Eunice viridis* can take place regularly once in October and once in November every year, although the dates fluctuate more widely than those of *Comanthus japonica*.

On the other hand, WHITMEE (1875) observed the swarming of *Eunice viridis* in January, February and even in March; from this

fact he emphasized the prime importance of the third quarter moon as such.

A more spectacular case is CASPERS' extensive investigation on the emergence of the imagos of the marine insect, *Clunio marinus* (1951). In Helgoland, this emergence shows a strict correlation with syzygy during the summer months. The adults live for only a few hours, but the unhatched larvae over-winter and emerge when the weather becomes warmer. CASPERS interfered with this natural course in two ways. (1) He succeeded in making them emerge in late autumn and in winter by heating the aquarium. (2) He performed the warming experiment at Verna in Bulgaria on the coast of the Black Sea, where the local time differs from that of Helgoland by 82 minutes. It was found that even in such unnatural emergence, the correlation with syzygy was evident and furthermore, the emergence at the two places took place at the same hour of the day according to the respective local times. For the above fact, no other explanation is possible but to say that syzygy itself is the direct cause.

The next point of interest is the interchangeability between the first and third quarter moons as stimulus to spawning in the Atlantic Palolo, *Eunice fucata*. Under ordinary circumstances, *E. fucata* spawns in connection with the third quarters of June and July. MAYER (1900, 1902*, 1908, 1909*) reports, however, that when the third quarter occurs very late in July, the spawning is shifted forward to the preceding first quarter. This situation somewhat resembles the *Comanthus* case since it suggests the presence of a border line of the breeding period beyond which spawning does not occur.

Concerning the relation of the spawning to the declination of the moon, CASPERS is the only worker who has taken up this point in connection with the emergence of imagos of *Clunio marinus* cited above (1951). However, CASPERS considers that the declination can only have a modifying effect on the syzygial rule in the induced emergence during winter.

In the present investigation on *Comanthus*, the declination was first considered simply as a substitute for the quarter moons, since

* quoted from Caspers' paper.

the southernmost and the northernmost positions of the moon practically coincide with the quarter moons during the period under consideration. However, on calculating the correlation coefficients and on examining the predictability for spawning, the declination turned out to be slightly superior to the quarter moons. In addition, the declination can furnish a further parallelism between the angle of declination and the degree of accuracy of the prediction, which may perhaps increase the weight of this factor beyond that of the quarter moons.

The reader may recall that WHITMEE discovered that the Atlantic Palolo swarms in connection with the third quarters of October and November but also with similar phases of the moon in January, February and March and he contended that this fact must indicate that the third quarter itself was the governing factor. Now in the case of *Comanthus* there is one more period during the year when the quarter moons and the maximum declinations coincide, which is March. It is worth special mention that, in March 1952, one female was found in the act of spawning. But very unfortunately this happened during the senior author's year and a half abroad and although the collecting staff of the Misaki Station was considerate enough to preserve the specimen, they neglected to record the date. Consequently, it is impossible to check the relation of this spawning to the dates of the maximum declination and the quarter moon.

The last intricate inter-relationship between the solar and lunar calendars in *Comanthus* spwaning is the fixedness of the spawning time of the female at 3 o'clock in the afternoon. As is the case with *Clunio* emergence as well as with the spawning in many other animals including the Palolo (CLARK and HESS, 1940 a), the reaction of the males almost always precedes that of the females and shows a wider spread on the time scale. In *Comanthus* the spawning of males is observed on the afternoon previous to that on which the females spawn, as well as about an hour before, at around 2 o'clock, or even later than 3 o'clock. On the contrary, year after year, the first spawning females are found between 3 and 3.10 but never before 3 o'clock. As a matter of fact, this is the most consistent feature of the spawning phenomenon. According to HYMAN (1955), Crinoidea seems to have many members which spawn at a definite hour of the day. *Lamprometra kleinzingeri* spawns at 7 a.m. (MORTENSEN,

1937) and *Heterometra savignyi* at about 3 p.m. in late August (MORTENSEN, 1938). BURY (1888) and SEELIGER (1893) also report egg-laying in *Antedon* and in others at about 7 in the morning.

However the study on the maturation division of *Comanthus* revealed that the eggs are set free from the chorions and begin the reduction divisions between 12.15 and 12.30 p.m. of the day of spawning (DAN, 1952). These observations might suggest the possibility that the alarm clock goes off at noon and the series of events following it may culminate in spawning at 3 p.m. more or less automatically. On the other hand, the senior author found in 1941 one female in which the eggs had already gone through the reduction divisions by 10 in the morning. which spawned at 3. There is no way of knowing whether the eggs in the ovaries of this female underwent maturation around 12 of the previous day and for some reason failed to be shed, or whether they went through maturation at some odd hour. But the fact that they were retained until 3 o'clock might contrariwise indicate that the animals receive a different signal at 3 o'clock specifically for spawning.

SUMMARY

1) The non-stalked crinoid, *Comanthus japonica*, spawns on a single afternoon in October at 3 o'clock every year.

2) From the observations made during the past 18 years, the yearly shift of the spawning dates seems to conform to the following rules:

a) The dates in 3 successive years are grouped into a triplet of 1st—3rd—1st quarters of the moon with the interval of 12.5 synodic months.

b) In the 4th year, a new triplet is started, the interval between the two triplets being 12.0 synodic months.

c) Once in 18 years, a large shift of the spawning date seems to occur.

3) Spawning dates can be correlated both with the moon quarters and with the maximum declination of the moon, the PEARSON correlation constants being 0.971 and 0.975, respectively.

4) During the period when the declination is larger than 22°, prediction on the basis of the declination is superior to that based

on the quarter moon, while when it is smaller than 22°, prediction on the basis of the quarter moon turns out to be better.

5) *Comanthus* spawning represents a very rare case in which a correlation is established between the reproductive cycle and the declination of the moon.

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THE DIFFERENTIATION *IN VITRO* OF THE ISOLATED ENDODERM UNDER THE INFLUENCE OF THE MESODERM IN *TRITURUS PYRRHOGASTER*

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INTRODUCTION

It was indicated by HOLTFRETER (1938 a, b) that the different endodermal regions of amphibian gastrula differentiate in isolation according to its prospective significance. In apparent contradiction to the result, BALINSKY (1938) observed the neighbourwise differentiation of the endodermal pieces transplanted in other endodermal regions at the neurula stage. Later it was pointed out by OKADA (1953, 1954 a, b) that the differentiation of the endodermal areas isolated from the *Triturus* gastrula is possible only when mesenchyme is present. More recently, he (1955 a, b, 1957) reported that the endoderm isolated from the neurula forms the pharynx when it comes to lie in mesenchyme, but forms the intestine when it is surrounded by the mesothelium. Data were presented which clearly evidenced the pluripotency of the endoderm even in the neurula stage. In spite of those important contributions, for understanding the precise mechanism of formation of the enteric system further systematic analysis is needed as to the relationship between the inherent tendencies of the endodermal areas and the morphogenetic effects exerted by the mesodermal areas. In the present report, the development of different regions of the endoderm isolated from the *Triturus* gastrula and neurula under the influence of the different

1) The author wishes to express her sincere gratitude to Prof. TUNEO YAMADA for the constant guidance and encouragement throughout the course of the present experiments, and for critical reading of the manuscript. Thanks are also due to Dr. O. NAKAMURA and Dr. Y. TAHARA of the Morphological Laboratory of Osaka Liberal Arts University for their valuable advices, and to Dr. T. S. OKADA of the Zoological Institute of Kyoto University for permission to mention some of his unpublished results.

parts of the mesoderm of the neurula is studied. Special efforts have been made to culture the explants long enough to allow the complete differentiation of endodermal tissues, which is required for a reliable identification of individual tissues.

Material and Methods

Embryos of *Triturus pyrrhogaster* were exclusivly used in the present experiments. The developmental stages referred to in this paper are those of Okada and Ichikawa (1947).

Endodermal source. The endodermal regions to be explanted were isolated according to Figure 1. The beginning gastrula (St. 11) was dissected to yield the piece, *e 1*, which lies dorsally to the pigmented groove, the first indication of the blastoporal invagination, the piece, *e 2*, which lies ventrally to the same, and the piece, *e 3*, locating at the center of the unpigmented area. All these pieces were taken from the superficial layer of the endoderm. From the neurula (St. 17, St. 18) the endodermal material of four different zones was prepared: The anterior region (*aE 1* in Fig. 1), the antero-ventral region (*vE 1* in Fig. 1), the middle region (*E 2* in Fig. 1), and the posterior region (*E 3* in Fig. 1). Also in those cases the superficial layer of the endoderm alone was isolated. According to the maps of presumptive areas of Balinsky (1947) and of Nakamura and Tahara[2] (oral communications), the isolated endodermal regions

Table 1. Prospective significance of endodermal areas

Endoderm isolated	Prospective significance
Gastrula : *e 1*	Pharynx, stomach and liver
〃 : *e 2*	Intestine
〃 : *e 3*	Intestine
Neurula : *aE 1*	Pharynx and stomach
〃 : *vE 1*	Stomach and liver
〃 : *E 2*	Intestine
〃 : *E 3*	Intestine

[2] Communication at the 25th Annual Meeting (1954) of the Zoological Society of Japan and personnal communications.

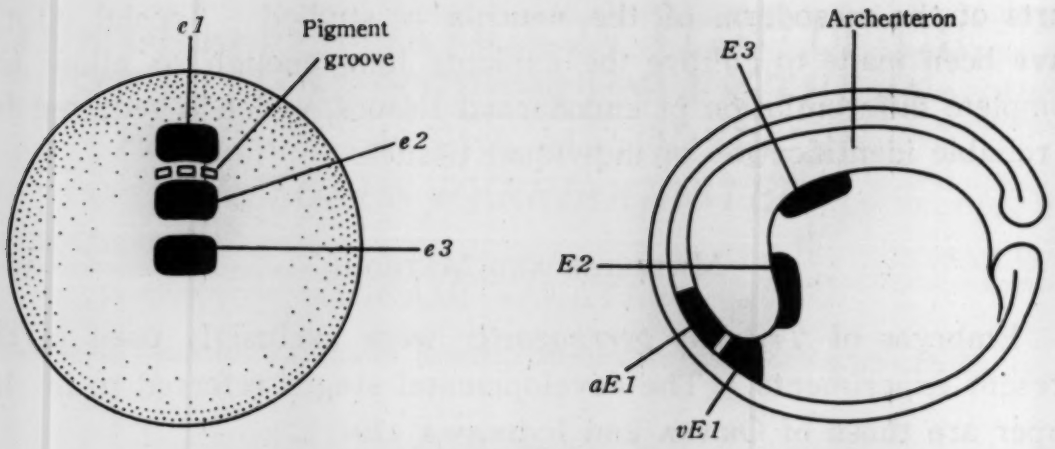

Fig. 1. Scheme of operation showing the areas of endoderm taken from the *Triturus* gastrula and neurula. Description in text.

are mainly destined to form the tissues given in Table 1 in the normal development.

Mesodermal source. The following regions of mesoderm were isolated from the embryos at stages 17 to 19 (Fig. 2); (1) The antero-lateral mesoderm, corresponding to the heart-forming area suggested by WILENS (1955); (2) The latero-ventral mesoderm, containing the presumptive blood island and lateral plate; (3) the presumptive trunk-somites; (4) The presumptive trunk-notochord. Often it was impossible to remove completely the neural plate cells adhering to the presumptive notochord.

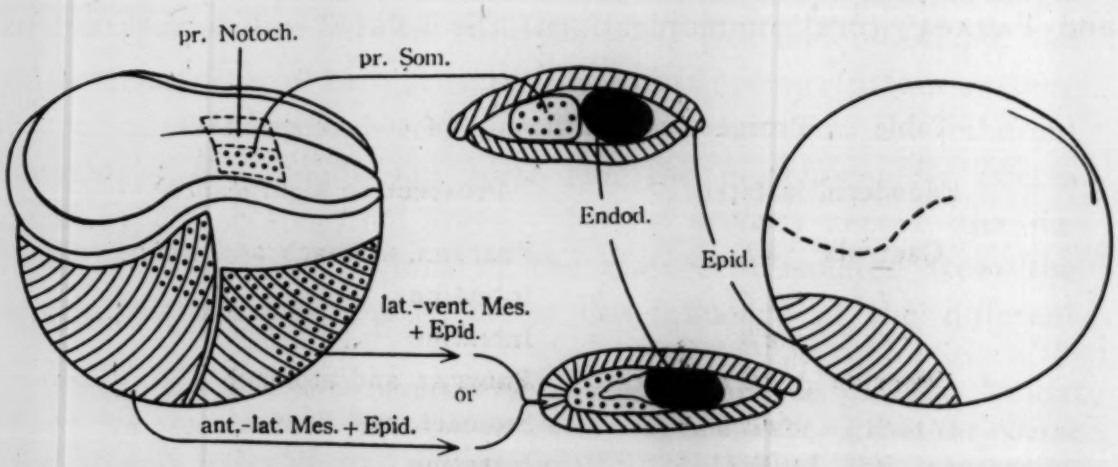

Fig. 2. Scheme of operation showing the mesodermal areas added to the endodermal piece and explants. Description in text.

Operation and cultivation. Aseptic procedures were used throughout. In the series with the presumptive trunk-somites, pieces of

endoderm and mesoderm were put between two pieces of the mesoderm-free ventral epidermis excised from the latest gastrulae (St. 15). In the series with the antero-lateral mesoderm or the latero-ventral mesoderm, instead of the mesoderm-free epidermis a piece of the epidermis lined with the corresponding mesoderm was used, and the endodermal piece was inserted into the ecto-mesodermal complex. In some series the presumptive trunk-notochord from the neurula was combined with the endoderm and mesoderm isolated.

The explants thus obtained were cultured at 18°C. for 38–75 days. Only a culture of such long duration enabled unequivocal identification of endodermal tissues, during which time the definite differentiation of all principal endodermal tissues sets in. The onset of definite differentiation of those tissues coincides temporally with disappearance of the bulk of yolk platelets. In the intestinal area, which is the slowest in development, the stage is reached *ca*, 40 days after the neurula stage at 18°C. Sterile HOLTFRETER's solution of pH 7.1–7.4 was used as the medium for operation and cultivation. Either sodium bicarbonate or Tris was used as the buffer. In the case of HOLTFRETER's solution buffered with sodium bicarbonate, it was found necessary to renew the culture medium once in three weeks because of gradual decrease of the pH value. The HOLTFRETER's solution buffered with Tris at pH 7.3–7.4 showed a stability in pH sufficient for the long term culture, making the replacement of the culture medium unnecessary. After culturing, all the explants were fixed in BOUIN's fluid, sectioned at 10 microns, and stained with MAYER's hemalum and picroblauschwarz.

Identification of individual endodermal tissues. The histological identification of the endodermal tissues in the present experiments was based on the following criteria.

Pharynx. The pharyngeal epithelium is composed of a double layer of squamous non-ciliated cells.

Oesophagus. The lining of the oesophagus consists of columnar epithelium which possesses ciliated cells. In the present culture similar epithelia without formation of cilia were sometimes encountered. These cases were also included under the oesophagus

in the present study.

Stomach. The epithelium of the stomach is characterized by ciliated or non-ciliated columnar epithelium which may be folded. The gastric glands are found underneath the epithelium. The nuclei of the stomach are somewhat elongated, and located in basal parts of the cells.

Liver. The cells are arranged in cords surrounded by some blood cells. The nuclei are spherical and lie near the center of each cell. Moreover, the nuclei and cytoplasm of the liver tend to stain less with MAYER's hemalum than those in the pancreas do.

Intestine. The intestine has a columnar epithelium with cuticular border and scattered goblet cells. The epithelium is characterized by ovoid nuclei lying in the basal third of each cell. The melanin pigment which is present in the intestinal epithelium between the nucleus and cuticular border of the normal layer in the intact larva under starvation can be used as the supplementary criterium.

Pancreas. The parenchyma of the pancreas consists of many acini arranged very closely. The nuclei lie near the bases of the cells forming the compound tubular glands.

RESULTS

I. Control series.

Series Ia. Explantation of endoderm alone with the epidermis. The endodermal pieces of four regions from gastrulae (St. 11) and neurulae (St. 17, St. 18), *i.e. e 1*, *e 3*, *aE 1* and *E 2*, were used (Fig. 1). Each piece was enclosed in two pieces of the epidermis from the latest gastrulae (St. 15) and cultured. In most of the explants the ectodermal envelope showed the tendency to extrude the endodermal piece within seven to fourteen days after explantation. Twenty-six, 28, 50 and 35 explants of endodermal areas, *e 1*, *e 3*, *aE 1* and *E 2* (Fig. 1) respectively, cultued for 16–35 days, were available for microscopic examination. The endodermal component of these explants showed no indication of differentiation, its cell being very large and containing a large amount of intact yolk platelets. Similar results were observed by OKADA (1954 a, 1955 a, 1957) in the explantation of the endodermal piece within the epidermal envelope.

Series Ib. Explantation of endoderm alone. From the regions of *e 1*, *e 3*, *aE 1* and *E 3* in Fig. 1, 38, 55, 23 and 24 pieces of the endoderm were taken respectively, and cultured for about 55 days. Most of the endodermal isolates remained unchanged throughout the culture. Microscopic examination revealed groups of yolk-rich cells devoid of any sign of differentiation (Fig. 3). Some of the explants contained pycnotic nuclei or indicated cytolytic conditions.

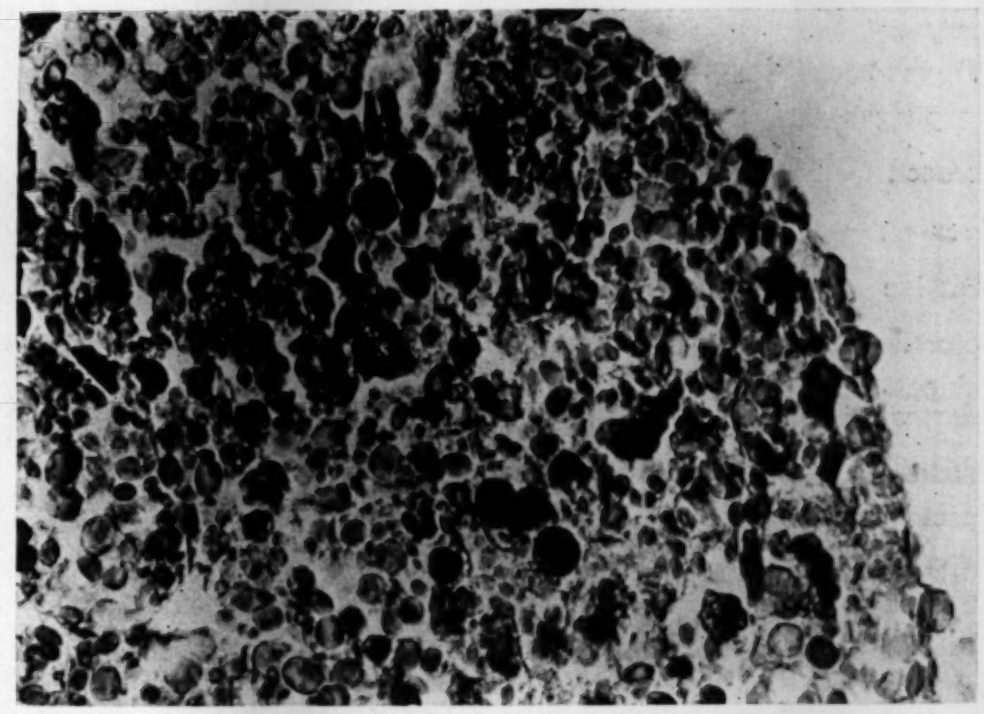

Fig. 3. Undifferentiated endodermal cells in a pure isolate of the endodermal piece, *e 3*, after forty-eight days of culture. ×*ca.* 340.

These facts demonstrate that the endodermal piece isolated from the gastrula or neurula, either with or without the epidermal covering, is unable to differentiate even if the culture is extended to 55 days.

II. Experimental series with mesoderm.

Series II a. Explantation of endoderm plus antero-lateral mesoderm. Each piece of seven different endodermal areas either from the gastrula or from the neurula was cultured with the epidermis and the antero-lateral mesoderm from the middle neurula. Most of the explants developed into expanded vesicles. The results of microscopic examination are summarized in Table 2.

Table 2. Differentiation in the explants with the endoderm and antero-lateral mesoderm

Endoderm isolated	Gastrula			Neurula			
	e 1	*e 2*	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants	31	22	47	34	39	23	16
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	4 (13%)	0	1 (2%)	2 (6%)	0	0	0
Gill pouches	0	0	0	0	0	0	0
Oesophagus	1 (3%)	0	0	0	0	0	0
Stomach	19 (61%)	5 (23%)	8 (17%)	15 (44%)	30 (77%)	17 (74%)	2 (12%)
Liver	25 (81%)	5 (23%)	11 (23%)	33 (97%)	39 (100%)	15 (65%)	0
Intestine	9 (29%)	18 (82%)	37 (79%)	3 (9%)	2 (5%)	6 (26%)	11 (69%)
Pancreas	4 (13%)	0	4 (9%)	1 (3%)	0	5 (22%)	1 (6%)
Unidentifiable endodermal tissue	0	0	1 (2%)	2 (6%)	0	0	2 (12%)
(Mesodermal)							
Muscle	0	0	0	0	0	0	1
Blood cells	31	21	32	33	34	23	8
Mesenchyme	31	22	47	34	39	23	16
Mesothelium	31	22	47	34	39	23	16
Heart	12	0	2	0	2	4	3

Mesodermal components. In all the explants, the mesothelium and mesenchyme were encountered. Blood cells also occurred very frequently. Formation of the heart was evidenced by pulsation during the culture as well as by the histological observation.

Endodermal components. In the series with the gastrula endoderm, the endodermal piece, *e 1*, containing the presumptive pharynx, stomach and liver, predominantly differentiated into the liver accompanied sometimes by the stomach, whereas the presumptive intestine (*e 2*, *e 3*) differentiated into the intestine at a very high frequency (Fig. 4). On the other hand, in the series with the neurula endoderm, the following results were obtained: (1) The principal product of piece *vE 1*, containing the presumptive stomach

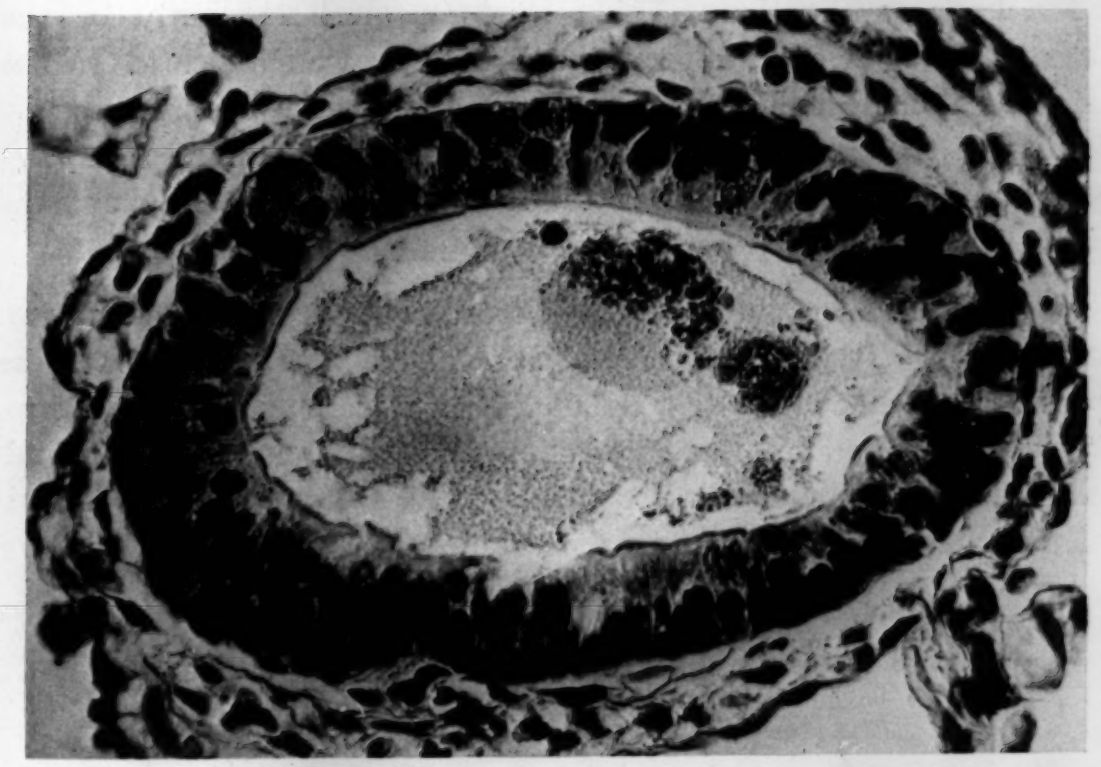

Fig. 4. An intestinal vesicle formed in an explant of the endodermal piece, *e 3*, combined with the antero-lateral mesoderm. Note the cuticular border. The vesicle is surrounded by mesenchyme. ×*ca.* 240.

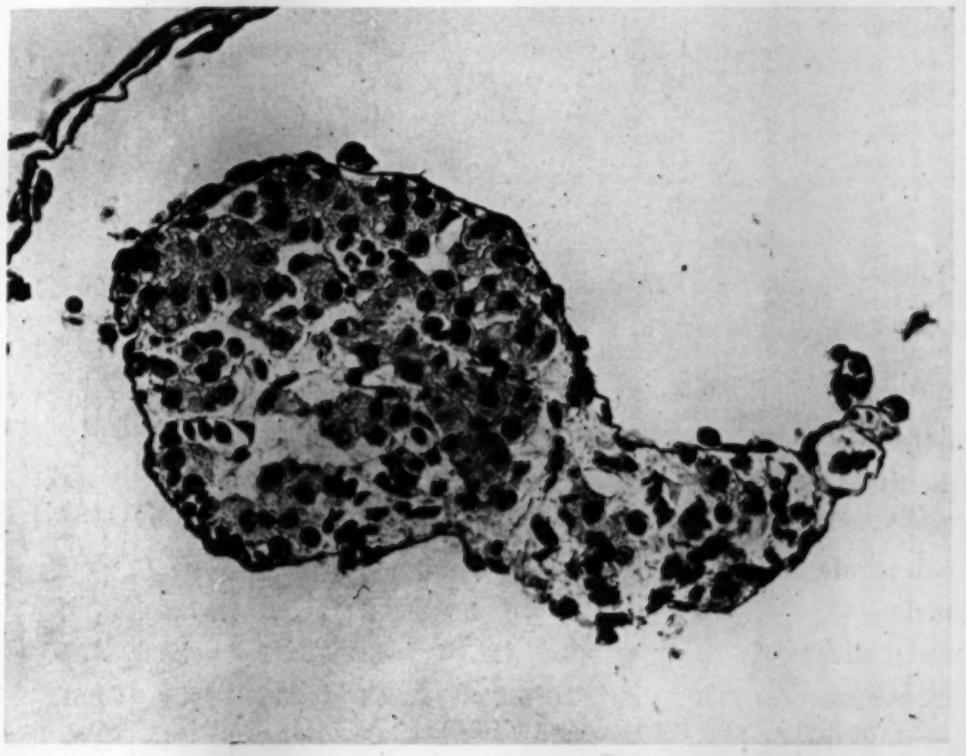

Fig. 5. Liver tissue formed in an explant combining the endodermal piece, *vE 1*, and the antero-lateral mesoderm. Note the blood cells and the mesothelial cover. ×*ca.* 160.

and liver, was the liver (Fig. 5) accompanied frequently by the stomach. (2) A good differentiation of liver was indicated in the endodermal materials, *aE 1* and *E 2*, which were taken from the vicinity of the presumptive liver. (3) Piece *E 3*, the presumptive intestine, mainly formed the intestine. The pharynx and pancreas occurred only rarely.

Series II b. Explantation of endoderm plus latero-ventral mesoderm. Each region of the endoderm described above was inserted into the epidermal envelope including the underlying latero-ventral mesoderm. Many of the explants formed swollen vesicles from which the limb rudiment was infrequently protruded. The results of the histological observation are summarized in Table 3.

Table 3. Differentiation in the explants with the endoderm and latero-ventral mesoderm

Endoderm isolated	Gastrula			Neurula			
	e 1	*e 2*	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants	41	26	43	36	31	33	34
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	0	0	0	2 (6%)	0	0	0
Gill pouches	0	0	0	0	0	0	0
Oesophagus	0	0	0	2 (6%)	1 (3%)	1 (3%)	0
Stomach	2 (5%)	1 (3%)	2 (5%)	9 (25%)	9 (29%)	2 (6%)	0
Liver	3 (7%)	1 (4%)	0	26 (72%)	11 (35%)	2 (6%)	0
Intestine	38 (93%)	24 (92%)	41 (95%)	21 (58%)	16 (52%)	26 (79%)	33 (97%)
Pancreas	4 (10%)	1 (4%)	0	3 (8%)	5 (16%)	0	0
Unidentifiable endodermal tissues	1 (2%)	0	0	0	13 (41%)	4 (12%)	1 (3%)
(Mesodermal)							
Muscle	1	0	2	0	0	0	0
Blood cells	40	26	42	36	30	31	33
Mesenchyme	41	26	43	36	31	31	34
Mesothelium	41	26	43	36	31	33	34
Heart	1	1	0	2	0	1	0

Mesodermal components. The explants always contained well-developed mesothelium and a large amount of blood cells. Mesenchyme was also found in all the explants, though small in quantity.

Endodermal components. Each piece from the gastrula (*e 1*, *e 2*, *e 3*) exclusively differentiated into the intestine, accompanied infrequently by the stomach, liver or pancreas. On the other hand, in the series with the endodermal piece of the neurula, an antagonistic relation between intestine and liver was evident: The frequency of intestine increased in the antero-posterior direction, whereas that of liver decreased in the same direction. Besides, the oesophagus, stomach and pancreas appeared at a low frequency in the explants with the endodermal pieces containing their presumptive material.

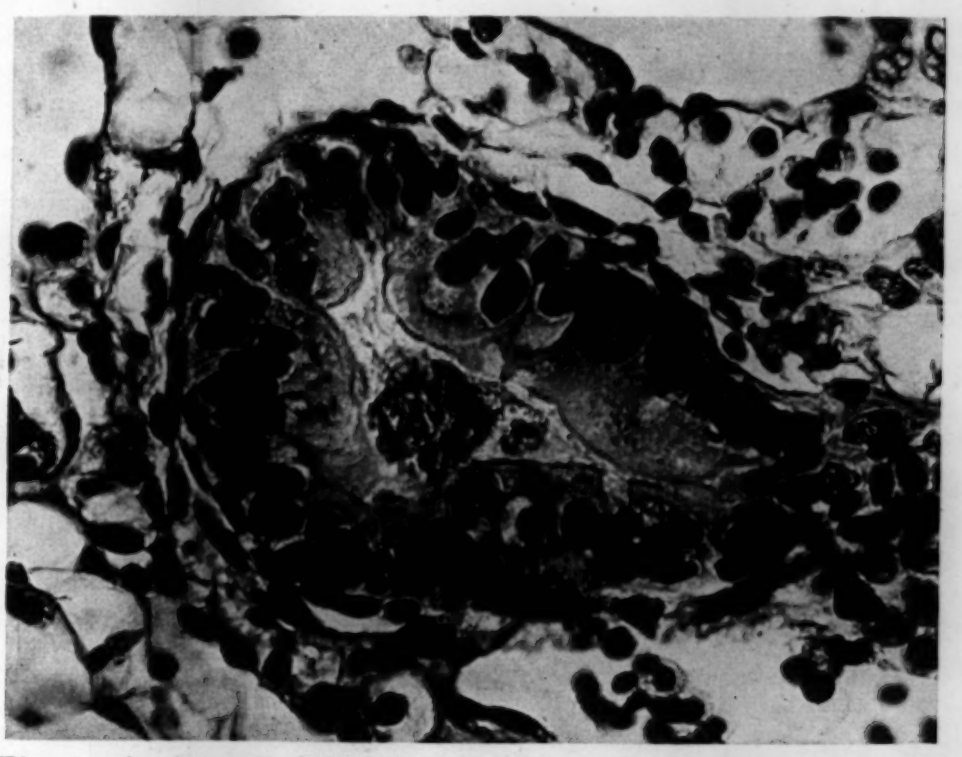

Fig. 6. An intestinal vesicle formed in an explant of the endodermal piece, *E 3*, combined with the latero-ventral mesoderm. ×*ca.* 240.

Series II c. Explantation of endoderm plus presumptive somites. Each of the endodermal regions from the gastrula and neurula combined with the presumptive trunk-somites was cultured in the epidermal envelope. During the culture, the explants developed into

rather swollen vesicles. The results of microscopic observation are shown in Table 4.

Mesodermal components. In contrast to the group with the antero-lateral mesoderm (Series II a) or the latero-ventral mesoderm (Series II b), the blood cells were found only very infrequently and in a small amount. Otherwise mesodermal structures were not much different from series II a, II b.

Table 4. Differentiation in the explants with the endoderm and presumptive trunk-somites

Endoderm isolated	Gastrula			Neurula			
	e1	*e2*	*e3*	*aE1*	*vE1*	*E2*	*E3*
No. of available explants	28	18	23	28	25	27	—
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	5 (18%)	0	0	4 (14%)	0	0	—
Gill pouches	3 (11%)	0	0	2 (7%)	0	0	—
Oesophagus	3 (11%)	0	0	0	0	0	—
Stomach	13 (46%)	4 (22%)	7 (30%)	16 (57%)	15 (60%)	12 (44%)	—
Liver	12 (43%)	4 (22%)	6 (26%)	23 (82%)	19 (76%)	14 (52%)	—
Intestine	11 (39%)	15 (83%)	17 (74%)	8 (28%)	12 (48%)	20 (74%)	—
Pancreas	3 (11%)	1 (6%)	0	1 (4%)	3 (12%)	7 (26%)	—
Unidentifiable endodermal tissues	0	0	0	0	1 (4%)	0	—
(Mesodermal)							
Muscle	2	0	0	4	0	1	—
Blood cells	4	2	8	1	10	3	—
Mesenchyme	28	18	23	28	25	27	—
Mesothelium	28	18	23	28	25	27	—
Heart	2	1	1	1	4	0	—

Endodermal components. Each of the endodermal pieces differentiated into the stomach, intestine and liver at a moderate frequency. A tendency to differentiate in conformity to the prospective significance of each endodermal region is apparent. The liver was

found accompanied by the stomach (Fig. 7) or intestine. The pharynx occurred only in a few explants which contained the presumptive pharynx (*e 1* and *aE 1*) as the endodermal material. In some explants, a small fragment of the pancreatic tissue was found in the vicinity of the intestine.

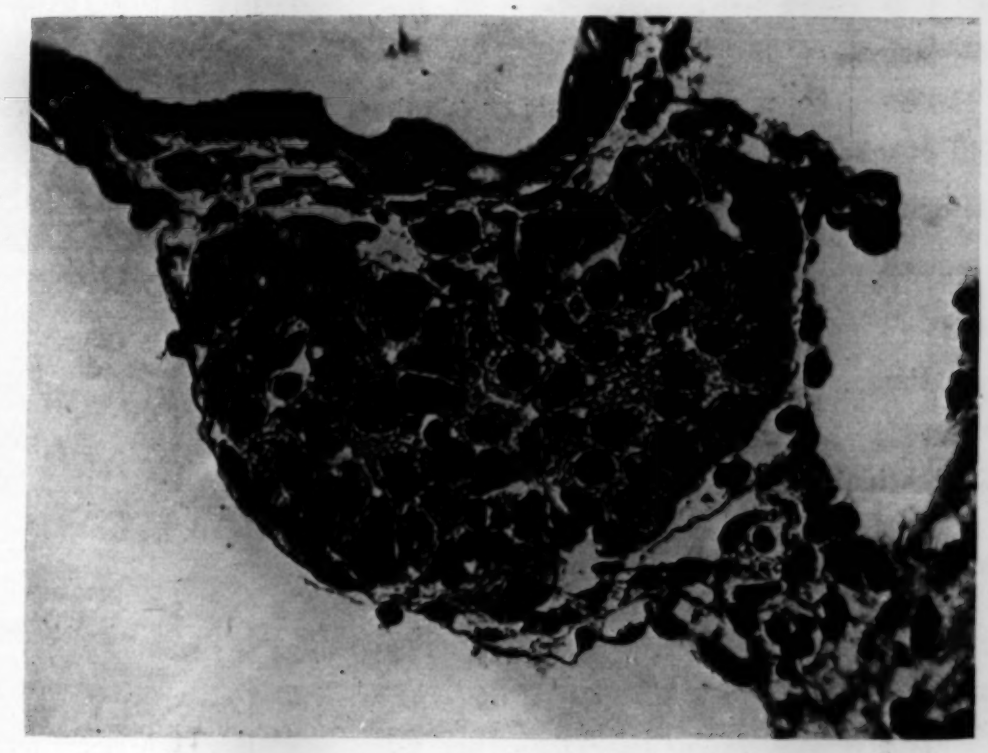

Fig. 7. Liver tissue in the vicinity of the gastric epithelium, formed in an explant of the endodermal piece, *e 1*, combined with the presumptive somites. ×*ca.* 240.

III. Experimental series with mesoderm and presumptive notochord.

Series III a. Explantation of endoderm, antero-lateral mesoderm and presumptive notochord. The presumptive trunk-notochord from the neurula was combined with the endodermal piece and antero-lateral mesoderm. The histological results are registered in Table 5.

Mesodermal components. The muscle, blood cells and heart were occasionally developed. The amount of mesenchyme produced appeared somewhat larger than in series II a, although its frequency remained approximately the same. The notochord was found in all the explants.

Table 5. Differentiation in the explants with the endoderm, antero-lateral mesoderm and presumptive trunk-notochord

Endoderm isolated	Gastrula			Neurula			
	e 1	*e 2*	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants	44	18	31	24	34	24	—
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	18 (41%)	3 (17%)	4 (13%)	16 (67%)	17 (50%)	1 (4%)	—
Gill pouches	9 (20%)	0	0	9 (37%)	11 (32%)	0	—
Oesophagus	13 (29%)	0	2 (6%)	3 (12%)	11 (32%)	3 (12%)	—
Stomach	23 (52%)	6 (33%)	14 (45%)	7 (29%)	21 (62%)	11 (46%)	—
Liver	33 (75%)	6 (33%)	15 (48%)	13 (54%)	17 (50%)	6 (25%)	—
Intestine	10 (23%)	7 (39%)	14 (45%)	8 (33%)	6 (18%)	13 (54%)	—
Pancreas	3 (7%)	1 (6%)	3 (10%)	2 (8%)	1 (3%)	1 (3%)	—
Unidentifiable endodermal tissue	2 (5%)	3 (17%)	2 (6%)	1 (4%)	1 (3%)	1 (4%)	—
(Mesodermal)							
Muscle	11	1	4	8	5	2	—
Notochord	44	18	31	24	34	24	—
Blood cells	39	6	8	21	25	19	—
Mesenchyme	44	17	31	24	34	24	—
Mesothelium	44	18	31	24	34	24	—
Heart	3	1	5	9	4	3	—
(Neural)							
Neural tissue	9	2	5	11	4	6	—

Endodermal components. Results deviating much from those of series II a without the presumptive notochord were obtained: (1) The presumptive intestine from the gastrula (*e 2*, *e 3*) formed the intestine in lower percentage. (2) The endodermal piece, *e 1*, from the gastrula containing the presumptive pharynx, stomach and liver, showed the differentiation into the pharynx in a relatively high percentage. (3) Low frequencies of liver were observed in the series with the endodermal piece from the neurula, in clear contrast to series II a in which endoderm was cultured with the presumptive

somites alone. (4) The endodermal piece, *aE 1* or *vE 1*, from the neurula produced the pharynx at a very high frequency.

Series III b. Explantation of endoderm, latero-ventral mesoderm and presumptive notochord. Pieces of the endoderm and presumptive trunk-notochord were reared within the epidermal jacket containing the latero-ventral mesoderm. Most of the explants grew into large expanded vesicles. Histological results are presented in Table 6.

Mesodermal components. All the explants revealed the notochord,

Table 6. Differentiation in the explants with the endoderm, latero-ventral mesoderm and presumptive trunk-notochord

Endoderm isolated	Gastrula			Neurula			
	e 1	*e 2*	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants	29	35	27	32	50	51	—
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	3 (10%)	0	1 (3%)	16 (50%)	1 (2%)	0	—
Gill pouches	0	0	0	8 (25%)	0	0	—
Oesophagus	0	0	0	23 (72%)	5 (10%)	1 (2%)	—
Stomach	6 (21%)	1 (3%)	2 (7%)	9 (28%)	13 (26%)	4 (8%)	—
Liver	7 (24%)	1 (3%)	2 (7%)	5 (26%)	18 (36%)	8 (16%)	—
Intestine	23 (79%)	31 (91%)	23 (85%)	11 (34%)	36 (72%)	33 (65%)	—
Pancreas	5 (17%)	1 (3%)	1 (4%)	1 (3%)	14 (28%)	1 (2%)	—
Unidentifiable endodermal tissue	0	5 (14%)	4 (15%)	0	3 (6%)	16 (31%)	—
(Mesodermal)							
Muscle	1	2	2	1	4	1	—
Notochord	29	35	27	32	50	51	—
Blood cells	29	34	19	28	46	43	—
Mesenchyme	29	35	27	32	50	51	—
Mesothelium	29	35	27	32	50	50	—
Heart	0	0	3	0	0	0	—
(Neural)							
Neural tissue	3	2	3	7	1	2	—

mesenchyme and mesothelium. Some of the explants produced nephric tubules, blood cells, muscle and limb rudiments as well.

Endodermal components. The results were generally similar to those of series II b, in which the presumptive notochord was excluded, with the exception of the series containing piece *aE 1*. In the series with piece *aE 1*, the differentiation into the intestine or liver was significantly suppressed, the pharynx and oesophagus being the principal products.

Table 7. Differentiation in the explants with the endoderm, presumptive trunk-somites and notochord

Endoderm isolated	Gastrula			Neurula			
	e 1	*e 2*	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants	26	18	30	29	21	26	18
No. of explants containing the following tissues							
(Endodermal)							
Pharynx	19 (73%)	8 (44%)	10 (33%)	28 (96%)	15 (71%)	9 (34%)	6 (33%)
Gill pouches	9 (35%)	3 (17%)	6 (20%)	22 (76%)	13 (62%)	3 (12%)	3 (17%)
Oesophagus	8 (31%)	1 (6%)	4 (13%)	0	6 (29%)	0 (23%)	1 (6%)
Stomach	9 (35%)	5 (28%)	14 (47%)	3 (10%)	9 (43%)	8 (31%)	4 (22%)
Liver	8 (31%)	3 (17%)	3 (10%)	0	12 (57%)	1 (4%)	0
Intestine	7 (27%)	11 (61%)	16 (53%)	0	7 (33%)	12 (46%)	9 (50%)
Pancreas	2 (8%)	0	2 (7%)	0	0	0	0
Unidentifiable endodermal tissue	0	1 (6%)	0	0	0	2 (8%)	2 (11%)
(Mesodermal)							
Muscle	18	12	12	28	8	10	6
Notochord	26	18	30	29	21	26	18
Blood cells	2	1	0	0	0	3	1
Mesenchyme	26	18	30	29	21	26	18
Mesothelium	15	18	30	25	21	26	14
Heart	0	0	0	2	0	0	0
(Neural)							
Neural tissue	6	4	9	12	8	8	7

Series III c. Explantation of endoderm, presumptive somites and notochord. Eighteen to thirty available explants were histologically examined. All the explants grew into swollen bodies of irregular contour. The results obtained in the present series are summarized in Table 7.

Mesodermal components. A large amount of mesenchyme was found within the explants, whereas the blood cells were revealed in few explants. Moreover, most of the explants contained muscle scattered in mesenchyme. Besides, the limb rudiment and melanophores were found in some explants.

Endodermal components. In the series with the endodermal material of the gastrula, piece *e 1* differentiated into the pharynx at a very high frequency. The pharynx was accompanied frequently by the oesophagus, stomach, intestine (Fig. 8) or liver (Fig. 9). On the other hand, piece *e 2* or *e 3* formed the intestine at a relatively high frequency (Fig. 10), and the pharynx at a relatively low frequency. Moreover, the oesophagus, stomach (Fig. 11), liver and pancreas occurred in some explants of series *e 2* and *e 3*. In

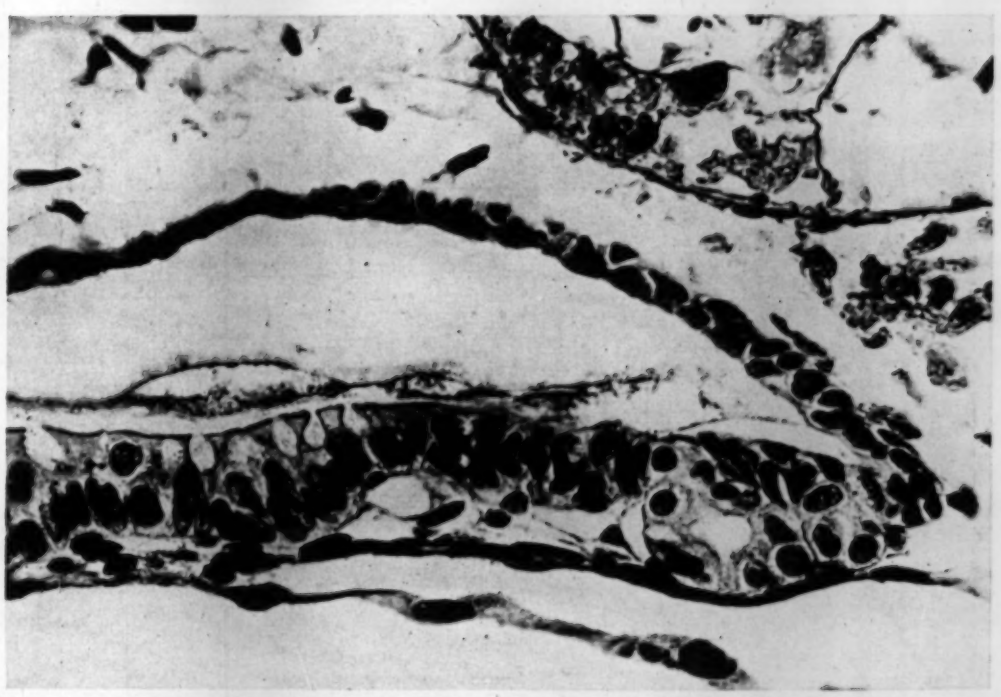

Fig. 8. Pharynx (above), stomach (below, right) and intestine (below left) forming a common vesicle in an explant containing the endodermal piece, *e 1*, presumptive somites and notochord. ×*ca.* 240.

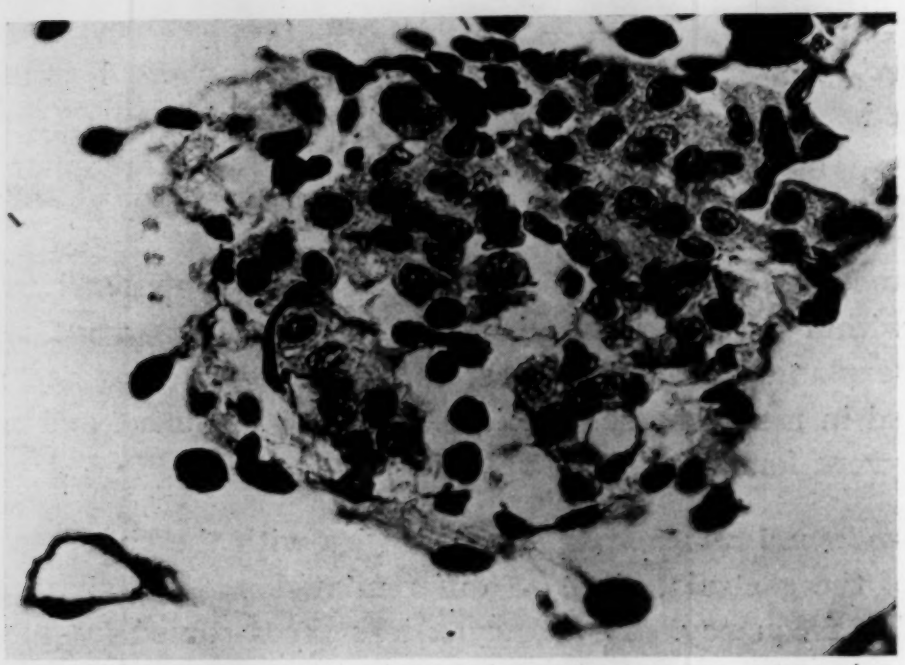

Fig. 9. A fragment of the liver tissue formed in an explant of the same series as illustrated by Fig. 8. ×*ca.* 240.

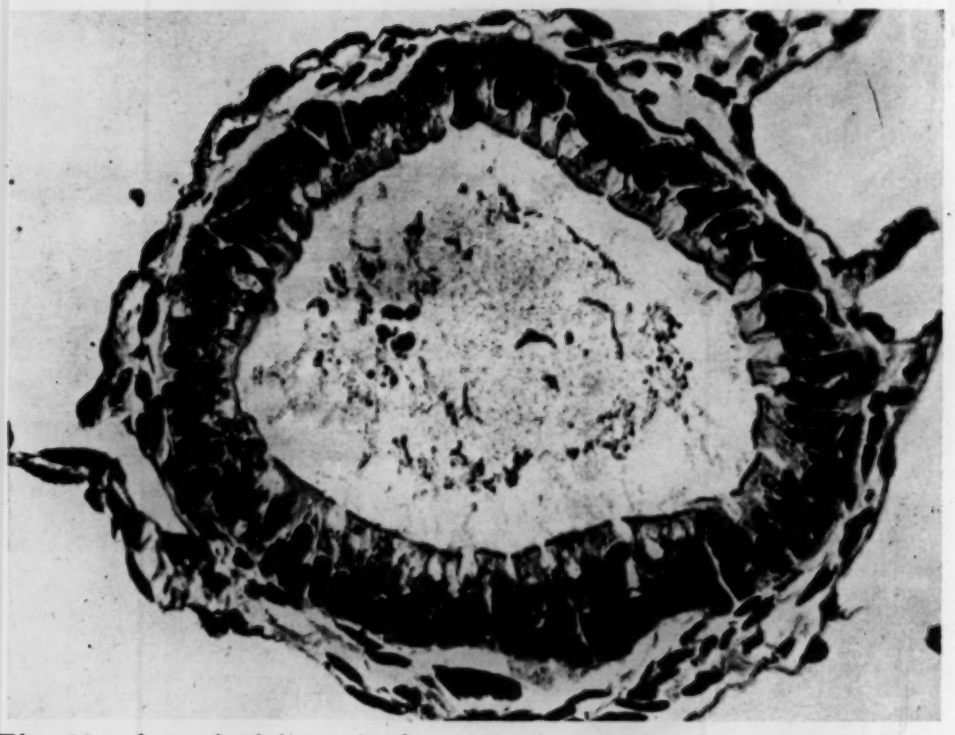

Fig. 10. A typical intestinal vesicle formed in an explant containing the endodermal piece, *e 3*, presumptive somites and notochord. Note goblet cells and cuticular border. ×*ca.* 240.

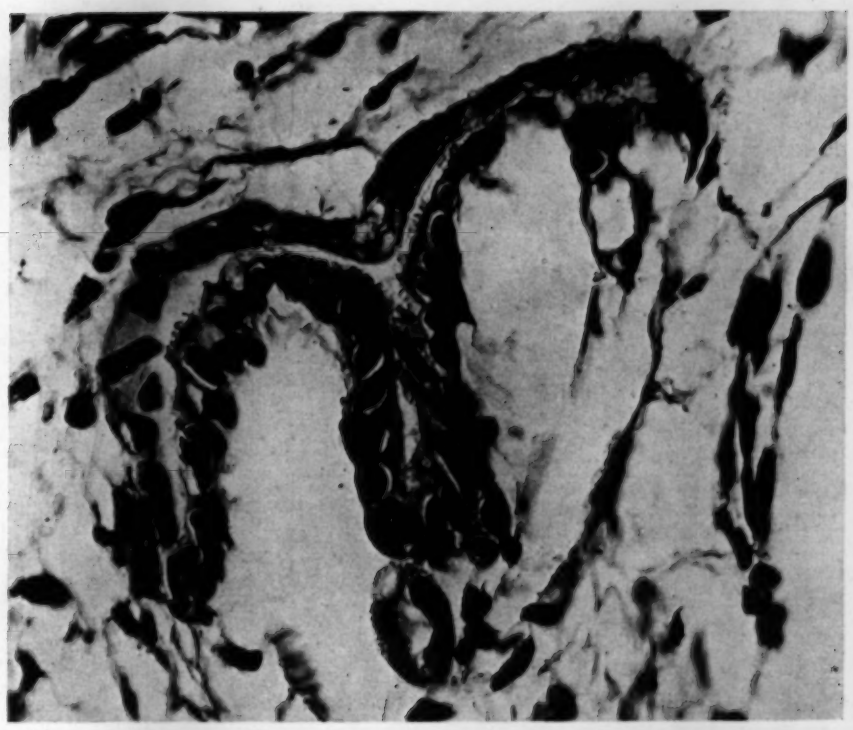

Fig. 11. Pharynx (above) and stomach (below) with the gastric glands differentiated in an explant of the same series as illustrated by Fig. 10. ×*ca.* 240.

Fig. 12. A pharyngeal vesicle accompanied by the gill pouches formed within mesenchyme. An explant of the endodermal piece, *aE 1*, cultured with the presumptive somites and notochord. ×*ca.* 310.

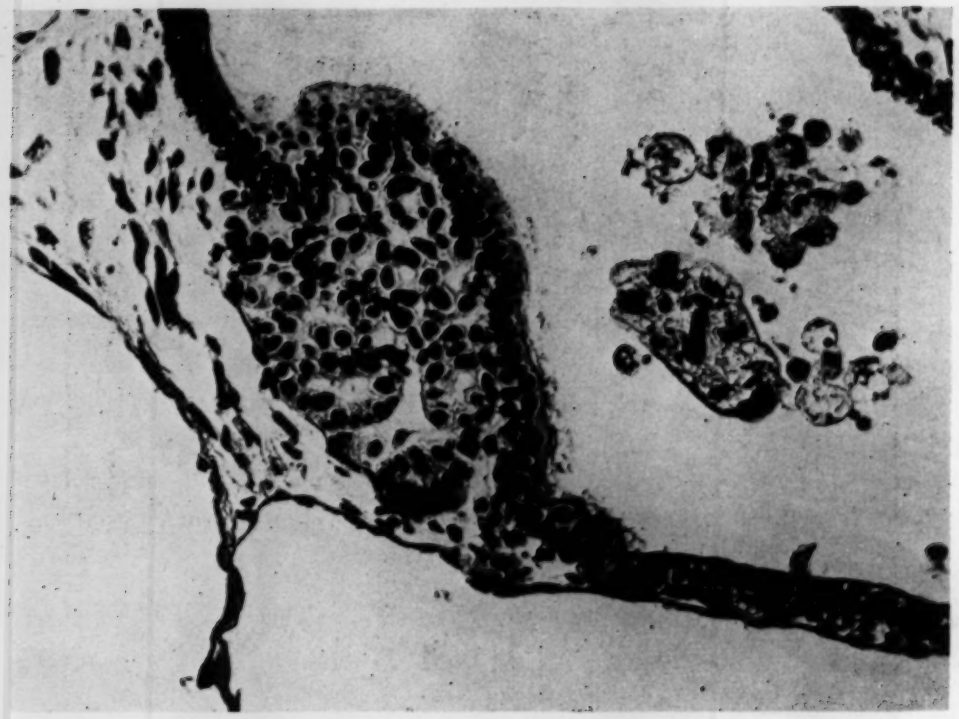

Fig. 13. A vesicle of typical stomach tissue possessing cilia and many gastric glands formed in an explant of the endodermal piece, *vE 1*, combined with the presumptive somites and notochord. ×*ca.* 160.

the series with the endoderm from the neurula, piece *aE 1* exclusively differentiated into the pharynx (Fig. 12), piece *vE 1* into the pharynx and liver at relatively high frequencies. In the series of the latter piece, the oesophagus and stomach (Fig. 13) were also found in some explants. On the contrary, the presumptive intestine (*E 2* or *E 3*) produced the pharynx, stomach and intestine in moderate percentages. Comparing with the gastrula series, the liver was found only in piece *vE 1* containing the presumptive liver. The pancreas was hardly produced in the present series.

Conclusions and Discussion

The technical difficulty in the study of the endodermal differentiation *in vitro* resides mainly in the slowness with which the germ layer is transformed into functional tissues with distinct morphological characteristics. Owing to the insufficient duration of culture many of the previous results obtained on the *in vitro* differentiation of the amphibian endoderm are either indefinite or not reliable, in

so far as differentiation is understood as formation of the distinct tissues out of the germ layer. In order to get a state of differentiation of the endoderm sufficient for a reliable identification of individual tissue, the explants must be cultured at least until the yolk platelets in the cells are almost completly utilized. Hence in the present study, a culture of very long duration was adopted (38 to 75 days at 18°C.). As shown in the microphotographs, the cells of the isolated endoderm thus cultured was practically free from yolk platelets, and revealed specific characters of the tissue.

From the present experiments, it is demonstrated that the endoderm isolated from both the gastrula and neurula stages differentiated into the tissues identifiable as pharynx, oesophagus, stomach, liver, intestine and pancreas, under the influence of the mesoderm. The array of endodermal tissues produced varied according to the combination of the endodermal and mesodermal areas isolated, and also according to the ages of the endoderm explanted. In the following the conditions which were found necessary for differentiation of individual structures will be summarized.

Differentiation of the pharynx. Although it is unquestionable that the pharyngeal tissue can be produced from all areas of the endoderm studied, a regional difference is demonstrable in the endoderm of both donor stages for the aptness to produce the tissue. As seen in Fig. 14, within the same mesodermal environment, the areas containing the presumptive pharynx (*e 1*, *aE 1*) have the highest frequency of the tissue. Especially under the condition which is favourable for the pharyngeal differentiation (series with the presumptive somites and notochord) the frequency of pharynx steadily decreases from the anterior to the posterior endodermal regions. On the other hand, the frequency of pharynx depends upon the kind of mesoderm added to the endodermal pieces. While the presumptive somites, antero-lateral mesoderm, and latero-ventral mesoderm gave only low frequencies of pharynx according to the order given, higher frequencies of the tissue were obtained in the series with the presumptive notochord added to one of those mesodermal areas. The pharynx differentiated in the highest percentage when the isolated endoderm was cultured with the presumptive somites and notochord. The nature of the environment derived from

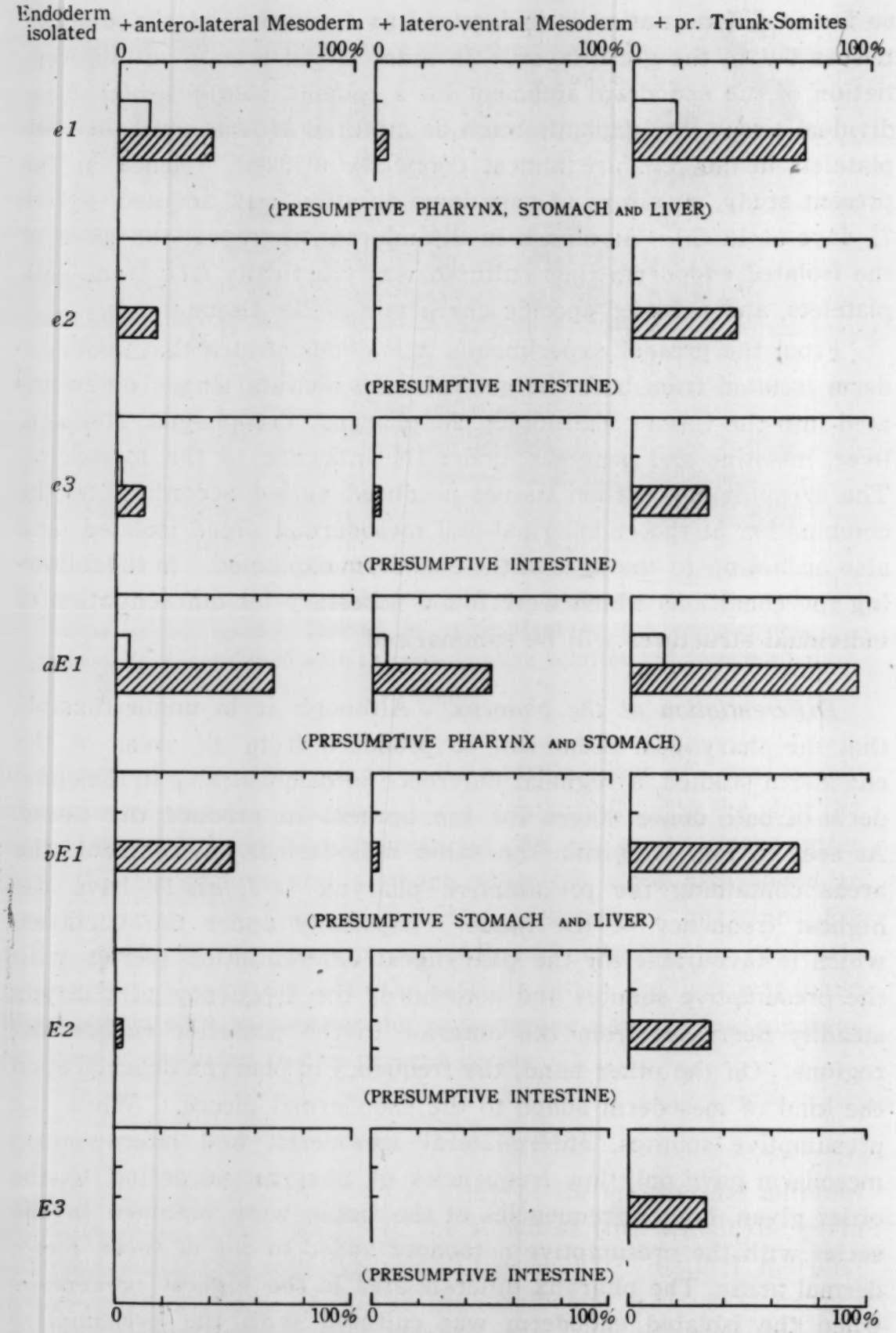

Fig. 14. Diagrams illustrating the frequencies of pharynx in various experimental series. Blank bars: Frequencies of the tissue in the series without the presumptive notochord. Shaded bars: Frequencies of the tissue in the series with the presumptive notochord. The frequency of the tissue is expressed by the percentage of the number of explants containing the tissue against the total number of explants in each series.

the mesoderm and presumptive notochord influenced not only the frequency of pharynx, but apparently also the amounts of the tissue formed, the amount roughly correlating to the frequency. With a few exceptions the pharynx was surrounded with mesenchyme. This conforms to the idea of OKADA (1955 a, b, 1957) that mesenchyme is responsible for the formation of pharynx.

Differentiation of the oesophagus. The frequency of the tissue formed in the explants was generally very low, although the tissue was produced from all tested areas of the endoderm either of the neurula or of the gastrula. The endodermal piece containing the presumptive pharynx and stomach of the neurula very frequently differentiated into the oesophagus together with the pharynx or stomach under the influence of the latero-ventral mesoderm and notochord. However, the latero-ventral mesoderm alone induced the formation of the oesophagus from the same endodermal region only at a very low frequency.

Differentiation of the stomach. Also the tissue was formed from all tested areas of the endoderm of both donor stages. When various endodermal pieces combined with the same mesodermal areas were compared, the piece containing the presumptive stomach most frequently gave rise to the stomach. In the majority of cases, the gastric tissue obtained in the present experiments was connected with the hepatic tissue. Hence there was a general tendency for parallelism of frequencies between these two tissues under various conditions. Thus the highest frequency for the stomach as well as for the liver was obtained in the series in which the presumptive stomach and liver were combined with the antero-lateral mesoderm. However, on addition of the presumptive notochord to the last mentioned combination, in spite of a strong decrease in the frequency of liver, only a slight, if any, decrease in the frequency of the stomach was observed (Table 6). The morphology of the gastric tissue found in the explants was considerably deviated from that of the corresponding normal epithelium.

Differentiation of the liver. Although all areas of the endoderm tested excluding region *E 3* produced the liver, there was a clear indication for the difference in capacity for producing the tissue

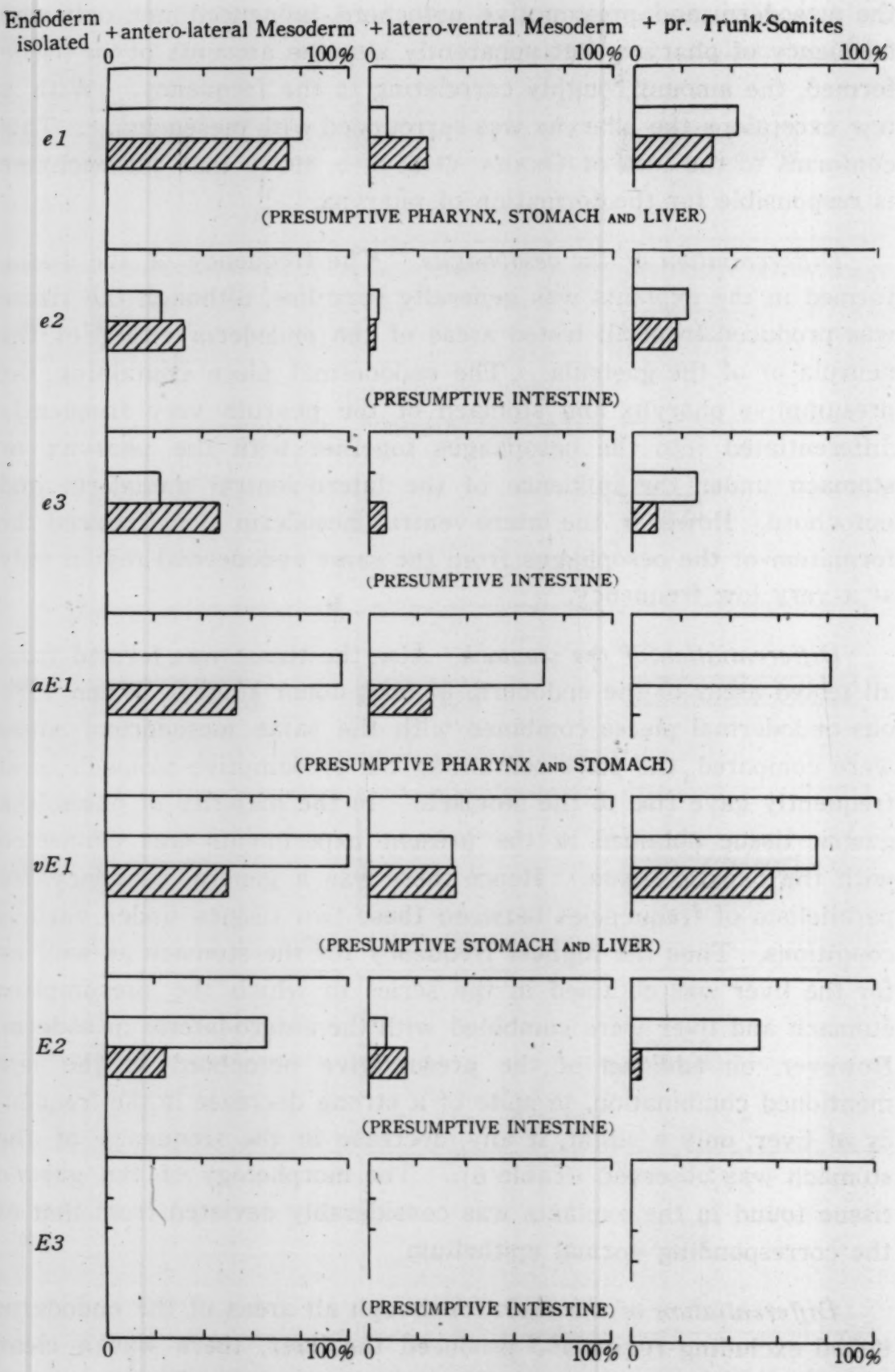

Fig. 15. Diagrams illustrating the frequencies of liver in various experimental series. For explanation see the legend of Fig. 14.

among various endodermal regions of both donor stages (Fig. 15). The anterior endodermal pieces show stronger tendencies for the liver differentiation than the posterior pieces, the gradient being especially clear in the series of the neurula endoderm combined with the antero-lateral mesoderm. The absence in region *E 3* of the liver forming capacity detectable with the present method fits in well with the antero-posterior gradient of the capacity. On the other hand, the difference in evoking the liver formation is detectable in mesodermal regions added to the endodermal pieces. The antero-lateral mesoderm was found most effective in this respect. Thus a culture of the anterior endodermal piece of the neurula in the presence of the antero-lateral mesoderm was followed by formation of well-developed liver at a very high frequency. Now, according to WILENS (1955), the antero-lateral mesoderm participates in formation of the heart in the normal development. Hence it was not unexpected to find a rather high frequency of the heart (Table 2) in the explants including the antero-lateral mesoderm. Further, in the normal development the same mesodermal area comes in direct contact with the endodermal area which subsequently forms the liver. Taken altogether these considerations make probable that an interaction between the antero-lateral mesoderm and liver endoderm is one of the epigenetic mechanisms operating in the morphogenesis of the liver. The suggestion conforms with the results of BALINSKY (1948) who pointed out an possible morphogenetic influence of the heart mesoderm upon the differentiation of the liver. In this connection it should be noticed that by combining various areas of endoderm with the mesoderm coated with epidermis, MANGOLD (1956) came to the conclusion that only the anterior endodermal areas give out an influence which favours the formation of the heart.

Differentiation of the intestine. As seen in Fig. 16, the formation of the tissue occurred in all endodermal areas tested. There was indication for the presence of regional differences in the competence of the endoderm to form the tissue, the posterior endodermal areas forming more frequently the intestine than the anterior areas under the same mesodermal environment do, although the difference was not as pronounced as in the case of the pharyngeal differentiation. The regional difference for the formation of the intestine is pro-

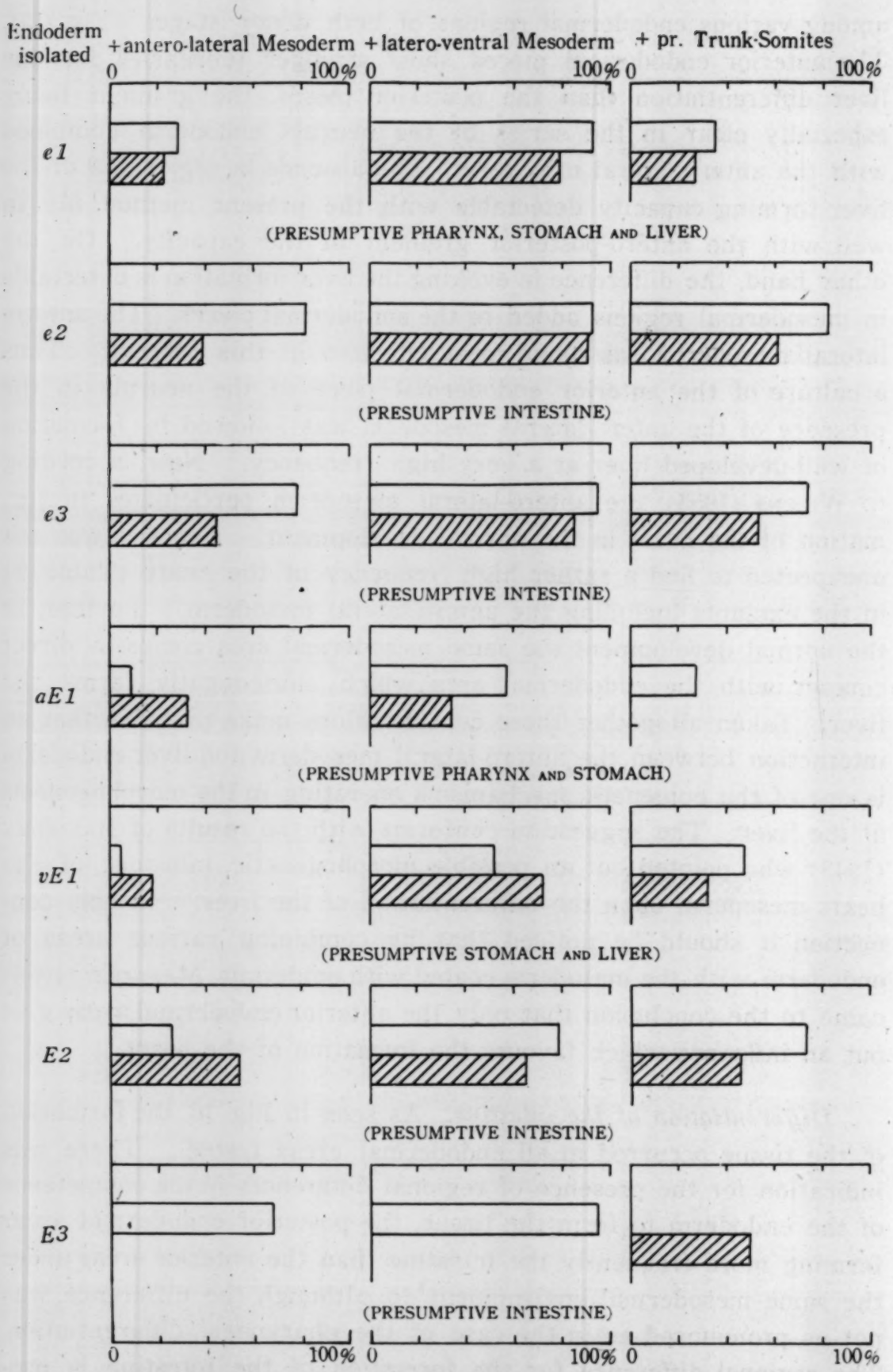

Fig. 16. Diagrams illustrating the frequencies of intestine in various experimental series. For explanation see the legend of Fig. 14.

nounced in the endoderm of the neurula, but is slight in that of the gastrula. On the other hand, a difference in enhancing the intestinal differentiation was indicated for various mesodermal areas. The tissue was most intensively enhanced by the latero-ventral mesoderm (Fig. 16). Thus the presumptive intestine of the neurula exclusively differentiated into the intestine under the influence of the latero-ventral mesoderm (Table 3). Addition of the presumptive notochord to other mesodermal areas tended to inhibit the intestinal differentiation of the endodermal component, although the inhibition was not always statistically significant. An exception was observed in the series in which the neurula endoderm was combined with the antero-lateral mesoderm, where addition of the presumptive notochord caused a significant rise in the intestinal frequency. However, much weight cannot be laid on the finding because of the low frequency of intestine.

Differentiation of the mesodermal components. The mesodermal components gave rise to tissues such as mesenchyme, mesothelium, blood cells, muscle, heart, etc., their frequencies and amounts varying according to the source of the mesoderm. The vacuolized notochord accompanied sometimes with small neural fragments was always observed whenever the presumptive notochord was present in the original explant. In the series without the presumptive trunk-notochord, the quantity of mesenchyme in each explant was rather small, but that of the mesothelium larger. On addition of the presumptive notochord the quantity of mesenchyme increased and that of the mesothelium decreased.

The formation of mesothelium in all explants of the series with the presumptive trunk-somites (Table 4) deserves attention, since the mesodermal area does not contain the lateral plate, as the other two mesodermal areas do. Further, the absence of muscle in most of the explants of the same series implies that the pathway of differentiation of the presumptive somites was considerably diverted from its normal course. The presence of the presumptive notochord in the comparable explant changed the situation (compare Table 4 and 7): The frequency of muscle was raised, that of mesothelium and bood cells slightly lowered. Also the amount of mesothelium in each explant appeared diminished. The plasticity of differenti-

ation of the mesoderm, and the effects of the notochord observed here conform well with the earlier observations made on the isolated mesoderm (YAMADA, 1940; MUCHMORE, 1951). In comparing the present data with that of the cited experiments, it should be pointed out that the stage of the mesoderm donor in the present experiments was rather late, so that a profound alteration in the differentiation pattern was not expected.

The significance of mesodermal areas. Considered from the effect on the development of endodermal tissues, some differences exist between the three mesodermal areas examined. The antero-lateral mesoderm was conducive to the differentiation of the liver. The highest frequency of liver was obtained when the antero-lateral mesoderm was combined with the endoderm containing the presumptive liver (see the first column of Fig. 15). In the presence of the latero-ventral mesoderm, the differentiation of the intestine was favoured, irrespective of the prospective significance of the endoderm (see the second column of Fig. 16). Since in the normal development the antero-lateral mesoderm comes in contact with the liver rudiment, and the latero-ventral mesoderm covers the intestine, the above observations suggest that the mesodermal area normally connected with a definite endodermal structure exerts a morphogenetic influence favourable for formation of the endodermal structure in question. On the other hand, the presumptive trunk-somites elicited the differentiation of the liver from the presumptive liver, and that of the intestine from the presumptive intestine (compare the third column in Fig. 15 with the same column in Fig. 16). However, the tendency to support the liver differentiation was weaker in the presumptive somites than in the antero-lateral mesoderm, and the tendency to support the intestine differentiation was weaker in the presumptive somites than in the latero-ventral mesoderm.

Addition of the presumptive notochord to one of the above mentioned mesodermal areas caused a definite change in the developmental pathways of the endodermal component: A significant enhancement of the pharynx frequency was caused when the presumptive notochord was added to the mesodermal component (Fig. 14). Especially clear was the effect in the series with the presumptive somites. In the neurula series, the enhancement of the

pharynx frequency was accompanied by the decline of the liver frequency. Also the intestine frequency was altered by addition of the presumptive notochord to the mesodermal environment. However, the facts that the effect was positive in some series and negative in others make the interpretation of the results difficult. In any case, the present results taken together speak positively for the morphogenetic effect of the presumptive notochord on the endodermal differentiation, in apparent contradiction to the statement made by OKADA (1953) that the presence of the notochord does not influence the outcome of the endodermal differentiation. It appears, however probable, that the effect of the notochordal material on the endoderm is exerted not directly but through the changes in differentiation of other mesodermal components.

OKADA (1955 a, b, 1957) reported that the endodermal pieces located in mesenchyme differentiated into the pharynx, and those coated with the mesothelium differentiated into the intestine. In the present experiments, the pharyngeal epithelium was found mostly located in mesenchyme, but infrequently inclosed with mesothelium. Further, the intestinal epithelium observed in our explants was mostly coated with the mesothelium, but occasionally (roughly in 10% of cases) surrounded with mesenchyme. It should be added that the interpretation of histological observation was sometimes ambiguous, owing to the occurrence of various situations intermediate between imbedding in mesenchyme and coating with the mesothelium. Thus, with some reservations our results as a whole confirm the above observations of OKADA. However, in view of the possibilities that the relation between endodermal and mesodermal tissues developed within the explant may be mutual rather than one-sided, and the differentiation of endodermal cells may be controlled by soluble agents in the internal space of the explants, it may be premature to rely too much upon the histological finding of the surface contact of the endodermal piece alone in discussing the controlling mechanisms of the endodermal differentiation.

Determination of the endoderm. From what has been stated above emerges the following picture: Although the endoderm itself has a very limited capacity for development when isolated from the mesoderm, the germ layer possesses clear regional differences already

before the gastrulation, which are demonstrable by comparing the differentiation capacities of various endodermal regions revealed under the influence of the given mesodermal areas. For instance, the presumptive pharynx of the beginning gastrula is able to form the pharyngeal vesicle significantly more frequently than the presumptive intestine under the comparable mesodermal environment. Thus, a clear indication is observed for the existence in the endodermal areas of the tendency to differentiate according to their own prospective significance. A similar tendency is present also in the endoderm of the neurula stage. Further, a gradual intensification of the differentiation tendency according to the prospective significance is suggested by the comparison of frequencies of pharynx and liver of the neurula series and those of the gastrula series. Coupled with the intensification of the differentiation tendency according to the prospective significance, an accentuation of the regional difference in producing pharynx and liver is apparent (compare the gastrula series with the neurula series in Figs. 14, 15). The situation for the formation of the intestine, the representative posterior structure, is different from what has been stated above concerning the differentiation of the pharynx and liver, the representative anterior structures. The tendency to differentiate the intestine from the presumptive intestine does not show significant increase during the developmental phase studied (compare *e 3* with *E 2* or *E 3* of Fig. 16), while there is clear indication of decrease in the tendency to differentiate the intestine from anterior endodermal pieces (compare *aE 1* and *vE 1* with *e 1* in Fig. 16). The comparison of the situation for the pharynx and liver with that for the intestine suggests that whereas the determination of the anterior endodermal structures involves an enhancement of the differentiation tendency in the anterior regions, the determination of the posterior structue involves a suppression of differentiation tendency in the anterior regions. These circumstances are related to the fact that in the gastrula series the intestine is formed at a high frequency from all areas of the endoderm studied, when the mesodermal environment is favourable for the formation of the tissue (see the gastrula series of the second column in Fig. 16), while the pharynx and liver are formed at high frequencies only from the anterior piece but at lower frequencies from the posterior pieces, even when the mesodermal environment

is favourable (see the gastrula series of the first and third columns of Fig. 14 and of the first and second columns of Fig. 15). The statement should not be understood to deny a regional difference in developing the intestine in the gastrula series, which is actually demonstrated (see the gastrula series of the first and third columns in Fig. 16), but it should only emphasize the relatively high frequencies of the intestine from the region containing no presumptive intestine in the gastrula series. In any case, the results presented show clearly that regional differentiation of the endoderm is progressing during the period between the early gastrula and middle neurula. This contradicts a suggestion made by OKADA (in press) that during the time of gastrulation no process of regional differentiation of the endoderm is proceeding, although his assertion that a gradient of morphogenetic potential exists in the endoderm of the gastrula is fully supported by the present data.

Progress of the development of the endoderm as controlled by the mesoderm. Not only regional specialization but also progress of development in the endoderm is dependent upon and controlled by the mesoderm. Evidence for it was first brought forward by the experiments of OKADA (1954 a, 1955 a, 1957) in which the endodermal piece of the neurula was wrapped with the epidermis and cultured. Even after 30 to 40 days of culture, the cells remained heavily yolk-laden, without the sign of cellular differentiation. In the present experiments a longer culture period was adopted, in order to exclude the possibility that the rather sudden disappearance of the bulk of yolk platelets in endodermal cells occurring normally at the stage corresponding to about 40 days of culture at 18°C. may be retarded in the explanted condition. The results of the experiments indicated that no perceptible progress of development occurred in the cells of isolated endoderm without the epidermal cover even after 55 days of culture at 18°C. The cells heavily packed with yolk platelets showed a size comparable to that of the normal endodermal cells of the neurula. In the developmental phase in question, since cell division would cause reduction of the cell size, failure to observe such reduction may suggest that cell divison was also suppressed in the isolated endoderm. Thus, under the experimental conditions developmental processes such as cell division, yolk platelets utiliza-

tion, and cytological and histoiogical differentiation in the endoderm are dependent upon the presence of the mesoderm in the vicinity. By contrast, in the isolated *ectoderm* of the gastrula, cell division, yolk utilization, and cellular differentiation of epidermal cells can proceed to some extent, though for differentiation of neural and mesectodermal tissues and for longer viability the presence of the mesoderm is required.

Summary

1. The endodermal pieces of various prospective significance were taken out from the beginning gastrula, or middle neurula, and explanted together with the various areas of the middle neurula. The embryos of *Triturus pyrrhogaster* were exclusively used.

2. In control series a small piece of the endoderm did not show any sign of development when explanted alone or cultured within the epidermis even after 55 days of culture. By contrast, differentiation of the endodermal pieces into definitive tissues, such as pharynx, oesophagus, stomach, intestine, liver and pancreas was observed when the piece was cultured with various areas of mesoderm for an extended period (38–75 days).

3. The type of endodermal tissues obtained was shown to be influenced mainly by the prospective significance of the endoderm as well as by the nature of the mesodermal areas added. To some extent, the developmental stage of the endoderm donor played a role too.

4. Although all endodermal areas tested showed the capacity to develop structures which are not in conformity with their own prospective significance, the tendency was observed that those areas produce more frequently the structures of their own prospective significance than the structures foreign to their prospective significance, in the same mesodermal environment.

5. Analysis of those or other data has suggested that a certain degree of determination is already present in the endoderm of the gastrula as to the regional differentiation, and that a progress occurs in the determination during the phase of gastrulation and neurulation. It has been further indicated that the pattern of determination of the anterior endodermal structures may be distinct from that of

the posterior endodermal structures, the former involving enhancement of corresponding differentiation tendencies in the presumptive areas, and the latter involving suppression of corresponding differentiation tendencies in the non-presumptive areas.

6. The formation of different types of endodermal tissues were favoured according to the areas of the mesoderm added. Thus, the antero-lateral mesoderm promoted the liver differentiation, the latero-ventral mesoderm the intestine differentiation, and the presumptive somites the liver and intestine differentiation. Addition of the presumptive notochord to any of those mesodermal areas resulted in enhancement of the frequency of pharynx, and, in the majority of cases, reduction of the frequency of liver.

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THE EFFECT OF DNP AND NaN_3 ON FERTILIZED EGGS OF THE SEA URCHIN WITH SPECIAL REFERENCE TO THE INDUCTION OF THE ABNORMAL CLEAVAGE*

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It has up to now been known that DNP and NaN_3 inhibit cleavage of the sea urchin egg (cf. KRAHL, 1950). The author has recently found that if fertilized sea urchin eggs are exposed to DNP- or NaN_3-sea water later than the streak stage, they often cleave directly into 4 cells after having been removed to ordinary sea water. The purpose of the present paper is to report on these experiments.

MATERIAL AND METHODS

Eggs of the following species of sea urchins were used as material: *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus*, *Temnopleurus toreumaticus* and *Anthocidaris crassispina*. Since all of these species gave essentially the same results, experimental data of the two former species are described in this paper.

A M/500 DNP solution in sea water and a M/10 NaN_3 solution in distilled water were taken as stock solutions. They were diluted to appropriate concentrations with sea water according to the purpose of the experiments.

In some experiments, the fertilization membrane and the hyaline layer were removed from the eggs in order to observe the mutual positions of blastomeres accurately.

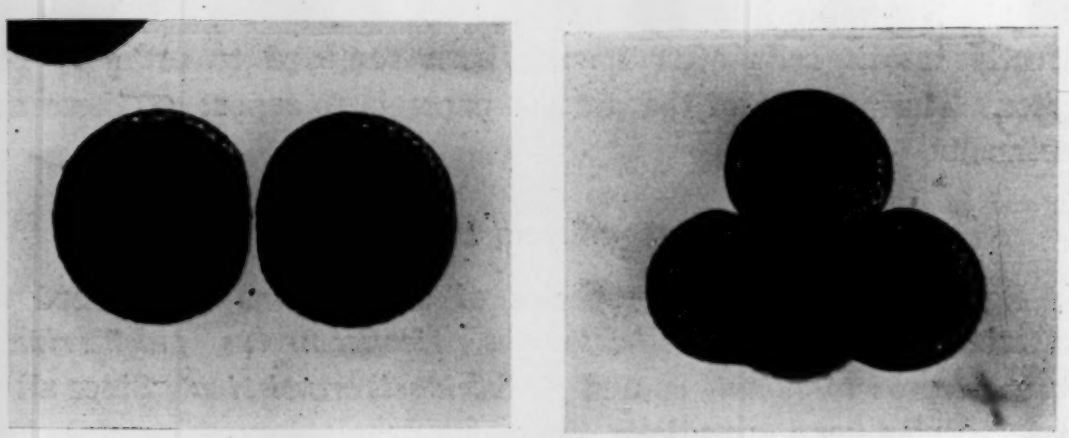
EXPERIMENTAL RESULTS

1) Effect of DNP-treatment

a) *Pseudocentrotus* eggs were fertilized and then put into a

* A part of this work was preliminarily reported in Japanese (KOJIMA, 1958).

M/1000 DNP-sea water at 10 minutes' intervals. The cleavage did not take place so long as they were kept in this medium. However, when these eggs were returned to ordinary sea water after 2 hours' DNP-treatment, they recovered from the cleavage-inhibiting effect (cf. CLOWES and KRAHL, 1936). When the DNP-treatment was applied before the streak stage, the eggs divided normally into 2 cells (Fig. 1 *a*), but when the treatment was done at a later stage, the eggs cleaved directly into 4 cells after recovery (Fig. 1 *b*). The mutual positions of the 4 blastomeres in the latter case are somewhat different from those in the 4-cell stage of the normal egg; that is, one blastomere is situated on the other three, which are arranged on one plane forming a triangle.



a *b*

Fig. 1. Cleavage of fertilized *Pseudocentrotus* eggs after the treatment with M/1000 DNP-sea water (17.5°C.).
a: egg put into DNP-sea water 40 minutes after fertilization.
b: egg put into DNP-sea water 70 minutes after fertilization.
These eggs were deprived of the fertilization membrane and the hyaline layer. In both cases, the eggs were returned to ordinary sea water after 2 hours' exposure to DNP.

Fig. 2 shows the relation between the time of DNP-treatment and the induction of the cleavage directly into 4 cells. In this figure the critical stage appears about 50 minutes after fertilization. This stage corresponds to the streak stage, which is followed by the prometaphase. The results of the DNP-treatment immediately before the first cleavage are omitted in this figure, because the eggs divided into 2 cells without being influenced by the treatment.

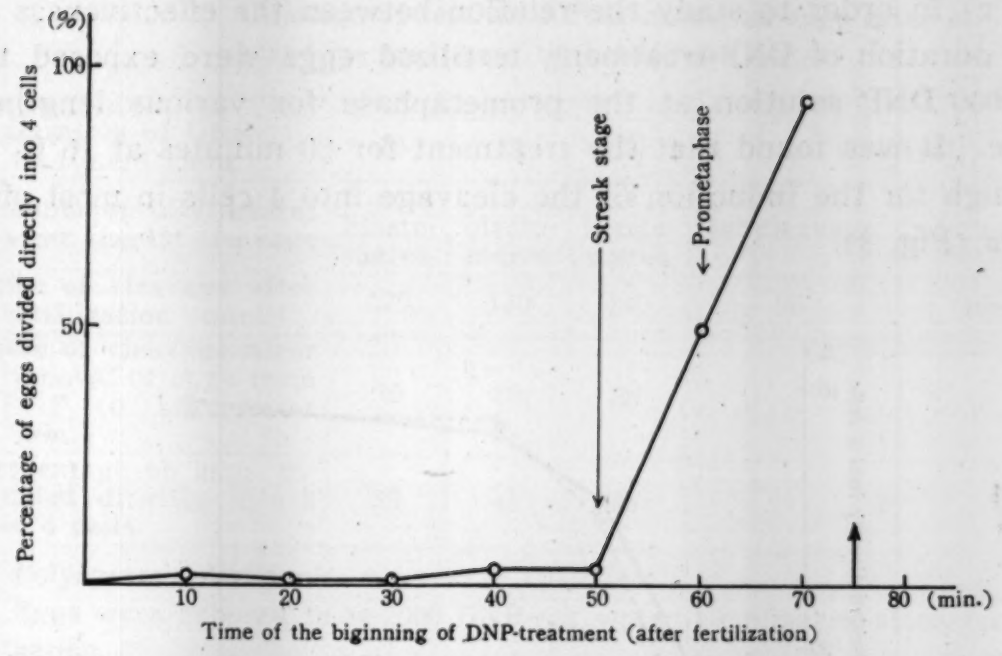


Fig. 2. Relation between the time of DNP-treatment and the induction of the direct division into 4 cells.——Fertilized *Pseudocentrotus* eggs were treated with M/1000 DNP-sea water for 2 hours (Temp. 17.5°C.).
Large arrow: the beginning of the first cleavage in the control egg.

b) In the next group of experiments, eggs were fertilized and then put into DNP-sea water of various concentrations at 10 minutes' intervals. The concentrations of DNP used were M/500–M/4000. The eggs were put back to ordinary sea water after 2 hours' DNP-treatment. It is seen from Table 1 that all the concentrations of DNP used were effective to induce the division directly into 4 cells, and that the most effective concentration was M/1000–M/2000.

Table 1. Relation between the concentration and the effectiveness of DNP

19°C.

Concentrations of DNP	Percentage of eggs which divided directly into 4 cells
M/ 500	80 (%)
M/1000	98
M/2000	96
M/4000	73

Pseudocentrotus eggs were fertilized, and, after 50 minutes, were treated with DNP-sea water of various concentrations for 2 hours.

c) In order to study the relation between the effectiveness and the duration of DNP-treatment, fertilized eggs were exposed to a M/1000 DNP solution at the prometaphase for various lengths of time. It was found that the treatment for 30 minutes at 16°C, was enough for the induction of the cleavage into 4 cells in most of the eggs (Fig. 3).

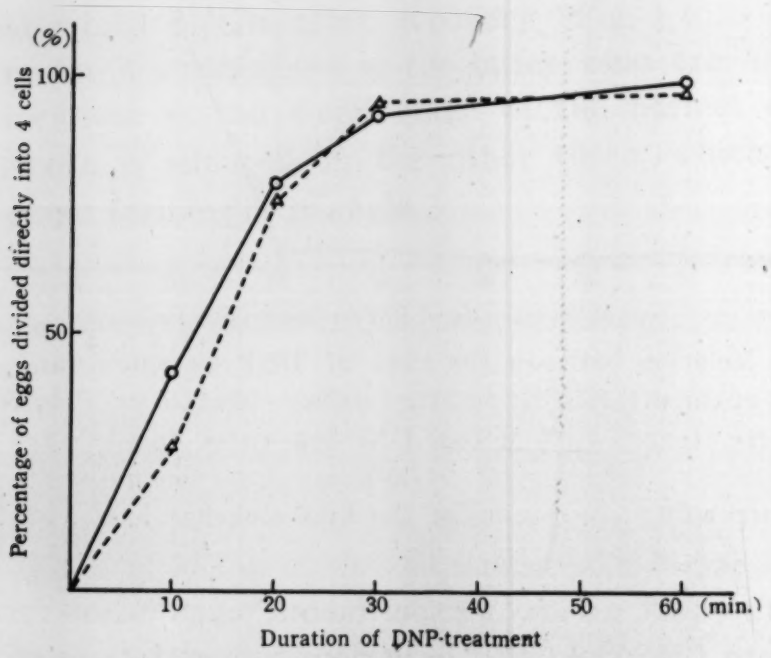


Fig. 3. Relation between the duration of DNP-treatment and the induction of the direct division into 4 cells.——*Pseudocentrotus* eggs were fertilized and then put into M/500 (−△−) and M/1000 (−○−) DNP-sea water 70 minutes after fertilization (Temp. 16°C.).

As is seen from Table 2, the DNP-treated eggs began to cleave into 4 cells within 30 minutes after they were returned to ordinary sea water. It is noteworthy that these eggs thus reach the 4-cell stage about 20–30 minutes faster than the normal eggs.

d) When eggs were treated with DNP later than the streak stage of the second mitotic cycle, they divided directly into 8 cells, forming a peculiar shape resembling two pyramids attached to each other at their bases as shown in Fig. 4. The arrangement of these 8 blastomeres were different from that in the normal 8-cell stage.

When eggs were exposed to the reagent later than the streak

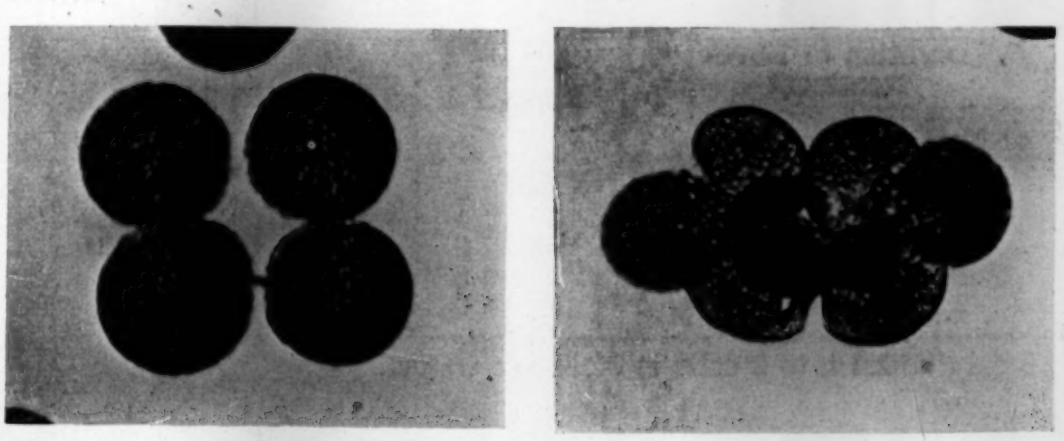
Table 2. Cleavage of fertilized *Pseudocentrotus* eggs after a short-time treatment with DNP-sea water

15°C.

Duration of DNP-treatment (min.)	10	20	30	0 (Control)	
Number of blastomeres after the 1st cleavage	3 or 4 blasto-meres	4 blasto-meres	4 blasto-meres	1st cleavage	2nd cleavage
Time of cleavage after fertilization (min.)	120	130	140	98	158
Time of cleavage after removal of eggs from DNP to sea water (min.)	30	30	30	—	—
Percentage of eggs divided directly into 3 or 4 cells	35	48	98	2*	

* Polyspermic division

Eggs were exposed to M/2000 DNP-sea water 80 minutes after fertilization.



a *b* *c*

Fig. 4. Cleavage of fertilized *Hemicentrotus* eggs after the treatment with M/1000 DNP-sea water (17°C.). —— *a*: egg put into DNP-sea water 85 minutes after fertilization.—— *b-c*: eggs put into DNP-sea water 95 minutes after fertilization.—— The egg shown in (*c*) was not deprived of the fertilization membrane and the hyaline layer. In both cases, the eggs were returned to ordinary sea water after 2 hours' exposure to DNP.

stage of the third mitotic cycle, most of them cleaved into 16 cells, while the rest divided into 8–15 cells. It is interesting to note that in the former case no micromeres were formed, while in the latter case sometimes several micromeres were produced.

2) Effect of NaN_3-treatment

Essentially the same results as those of DNP were obtained through the use of NaN_3. Table 3 shows one of the results with *Hemicentrotus* eggs. From this table, it may be recognized that the eggs divided directly into 4 cells when they were treated with NaN_3 later than the streak stage of the first mitotic cycle.

Table 3. Percentage of the direct cleavage into 4 cells in fertilized *Hemicentrotus* eggs after treatment with NaN_3-sea water

17°C.

Time when eggs were put into NaN_3-sea water (after fertilization)	30 min.		40 min.		50 min.		60 min.	
Duration of NaN_3-treatment → Concentrations of NaN_3 ↓	1 h	2 hs	1 h	2 hs	1 h	2 hs	1 h	2 hs
M/ 100	3	1	5	8	12	15	28	79
M/ 200	5	3	4	4	17	35	85	92
M/ 400	3	5	3	5	21	40	94	98
M/ 800	6	4	3	6	76	88	*	*
M/1600	4	3	9	32	95	92	*	*

* Eggs divided into 2 cells in NaN_3-sea water.

Discussion

It has been found that fertilized eggs divide directly into 4 cells if they are treated with DNP or NaN_3 later than the streak stage. The concentrations of DNP and NaN_3 employed in the present study are so high that these reagents can not be considered to act as uncoupling agents. There might be a possibility that DNP and NaN_3 induce the precocious division of the centrosome or the formation of the astral center at the prometaphase of the eggs.

Recently, Mazia and Zimmerman (1958) reported that the cleavage of *Strongylocentrotus* eggs is blocked reversibly by mercaptoethanol (monothoethylene glycol) and if the blocked eggs are left in mercaptoethanol sea water until the controls have completed the second division, they divide directly into 4 cells when they are re-

turned to sea water. It is interesting to note that the shape of the egg which has divided directly into 4 cells after mercaptoethanol-treatment is quite similar to that of the egg which has cleaved after DNP- or NaN_3-treatments. Further investigations, however, are necessary to discuss the modes of action of these reagents and to analyse the mechanism of this peculiar mode of cleavage.

Summary

1. Fertilized eggs of the sea urchin were treated with DNP-sea water (M/500–M/4000) or with NaN_3-sea water (M/100–M/400) for more than 30 minutes at various stages. When the treatment was carried out before the streak stage, the eggs divided into 2 cells after they were returned to sea water, but if the treatment was done later than the streak stage, the eggs divided directly into 4 cells. A similar procedure at the 2-cell or 4-cell stage induced division directly into 8 cells or 16 cells, respectively.

2. The DNP- or NaN_3-treated eggs reached the 4-cell stage 20–30 minutes faster than the normal egg.

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STUDIES OF THE METAMORPHOSIS IN INSECTS

IV. INHIBITION OF PUPATION BY CARBON DIOXIDE IN THE MATURE LARVA OF *DROSOPHILA MELANOGASTER*

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INTRODUCTION

In the previous study it was shown that deficient oxygen inhibits pupation in *Drosophila melanogaster* (TAKAOKA, 1959). In oxygen lower than 3.0%, pupation was suppressed, but at levels higher than 3.5%, no significant inhibition was observed. Carbon dioxide may also be a controlling factor of the metamorphosis in insects. That carbon dioxide is one of the general environmental factors affecting the biological phenomena has recently been emphasized (LOOMIS, 1958). In order to test the effect of carbon dioxide on pupation, pupation of mature larvae of *Drosophila melanogaster* was studied in various concentrations of carbon dioxide.

The writer wishes to express his sincere thanks to Prof. TUNEO YAMADA for his valuable criticism. Special thanks are also due to Prof. TAKESHI MORI for his valuable advice and to Mr. SADAO MATSUO of Institute of Earth Science, Faculty of Science for carrying out analysis of the gas contained in cavities of pumpkins.

MATERIAL AND METHOD

Pupation of mature larvae of *Drosophila melanogaster* was studied in gas mixtures with various carbon dioxide, oxygen and nitrogen tensions. According to the previous study (TAKAOKA, 1959), the tension of oxygen used in the following experiments (5–21%) is sufficient for pupation if no carbon dioxide is present.

1. Experimental animal. The material used was mature larvae of *Drosophila melanogaster*. The method of selection and the definition of "mature larvae" are given in the previous paper (TAKAOKA, 1959).

2. Gas mixtures. As the gaseous environment for larvae, carbon dioxide and oxygen were mixed with nitrogen under one atmospheric pressure. Compositions of the gas mixtures prepared are shown in Table 1.

Table 1. Compositions of gas mixtures under one atmospheric pressure

Constituent gases	Volume fraction (%)																	
	Group A							Group B							Group C			
O_2	5%							10%							21%			
CO_2	3	5	10	20	30	40	50	10	20	30	40	50	60	70	30	40	50	
N_2	92	90	85	75	65	55	45	80	70	60	50	40	30	20	49	39	29	

For mixing gases under one atmospheric pressure the apparatus shown in the previous paper (TAKAOKA, 1959) was modified; A device for introducing carbon dioxide was connected with the apparatus, and water for forcing gas out was replaced with 1/10 N sulphuric acid.

3. Exposure of larvae to gas mixtures. The technique adopted was as follows: Ten larvae were placed in a glass tube of about 6 cm. in length and 1 cm. in diameter, and the glass tube was closed at one end with gauze and the other end with moist absorbent cotton covered with gauze. Five such tubes were hung in a glass cylinder of about 3,600 cc. capacity. Before a gas mixture was introduced into the cylinder, the air contained in the cylinder was drawn for 2 minutes with a vacuum pump, and the cylinder was kept in vacuous condition for about 5 minutes to remove the air retained in the tracheal tubes of larvae as much as possible. The amount of mixed gas used in one series of experiment was about 7,000 cc. under one atmospheric pressure. Excessive gas was let out through the stopcock of the cylinder. Throughout the procedure, experimental animals were kept at 25°C.

4. Control experiment. The control experiment was done in the mixtures of 5, 10 and 21% oxygen with nitrogen. Before each run, the cylinder was evacuated as in the experimental series. Evacuation for 5 minutes did not reveal any effect on pupation.

Experiment

A population of mature larvae were exposed to mixtures of 5, 10 and 21% oxygen with carbon dioxide of graded concentrations, and the frequency of pupae were counted every 2 hours for 24 hours.

A. Exposure to mixtures of 5% oxygen with 3, 5, 10, 20, 30, 40 and 50% carbon dioxide at 25°C. (Fig. 1)

At levels higher than 30% carbon dioxide, significant suppression of pupation was observed. The number of pupae increased gradually through the whole course of experiment in the series with 3, 5, 10, 20, 30 and 40% carbon dioxide. In the series with 50% carbon dioxie, a low incidence of pupation was reached within 2

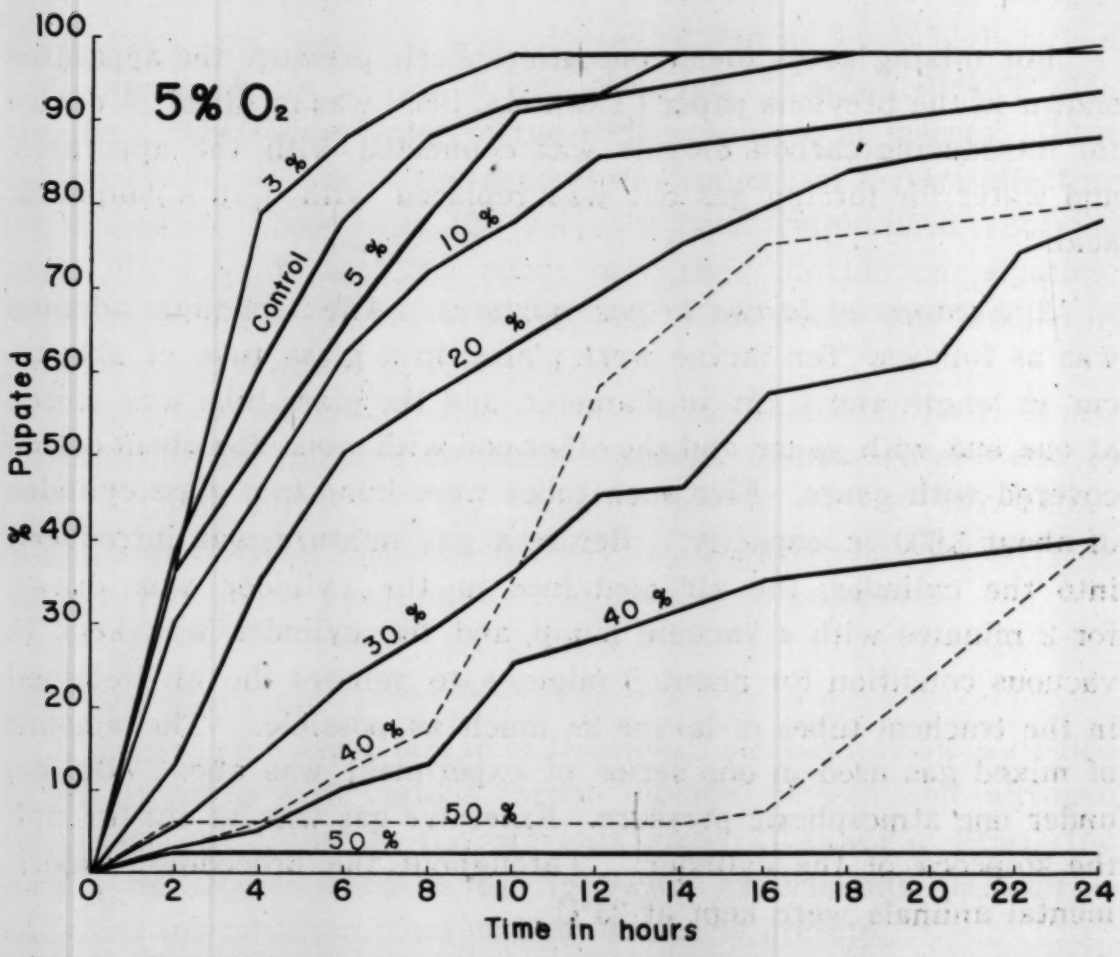

Fig. 1. Pupation of mature larvae of *D. melanogaster* kept in mixtures of 5% oxygen with various concentrations of carbon dioxide and nitrogen during the course of 24 hours (25°C.)

——: Completely pupated larvae

- - - -: Completely plus incompletely pupated larvae

hours, which remained unchanged later on. Incompletely pupated individuals (TAKAOKA, 1959) appeared in the series with 40 and 50% carbon dioxide.

B. Exposure to mixtures of 10% oxygen with 10, 20, 30, 40, 50, 60 and 70% cardon dioxide at 25°C. (Fig. 2)

The strong suppression of pupation was observed at the level of carbon dioxide higher than 40%. In the series with 50, 60 and 70% carbon dioxide, within first 2–4 hours a small number of individuals pupated, but no more pupation occurred later. During the whole course of experiment, the number of pupae increased gradually in the series with 10, 20, 30 and 40% carbon dioxide. Incomplete pupae appeared in the series with 40, 50, 60 and 70% carbon dioxide.

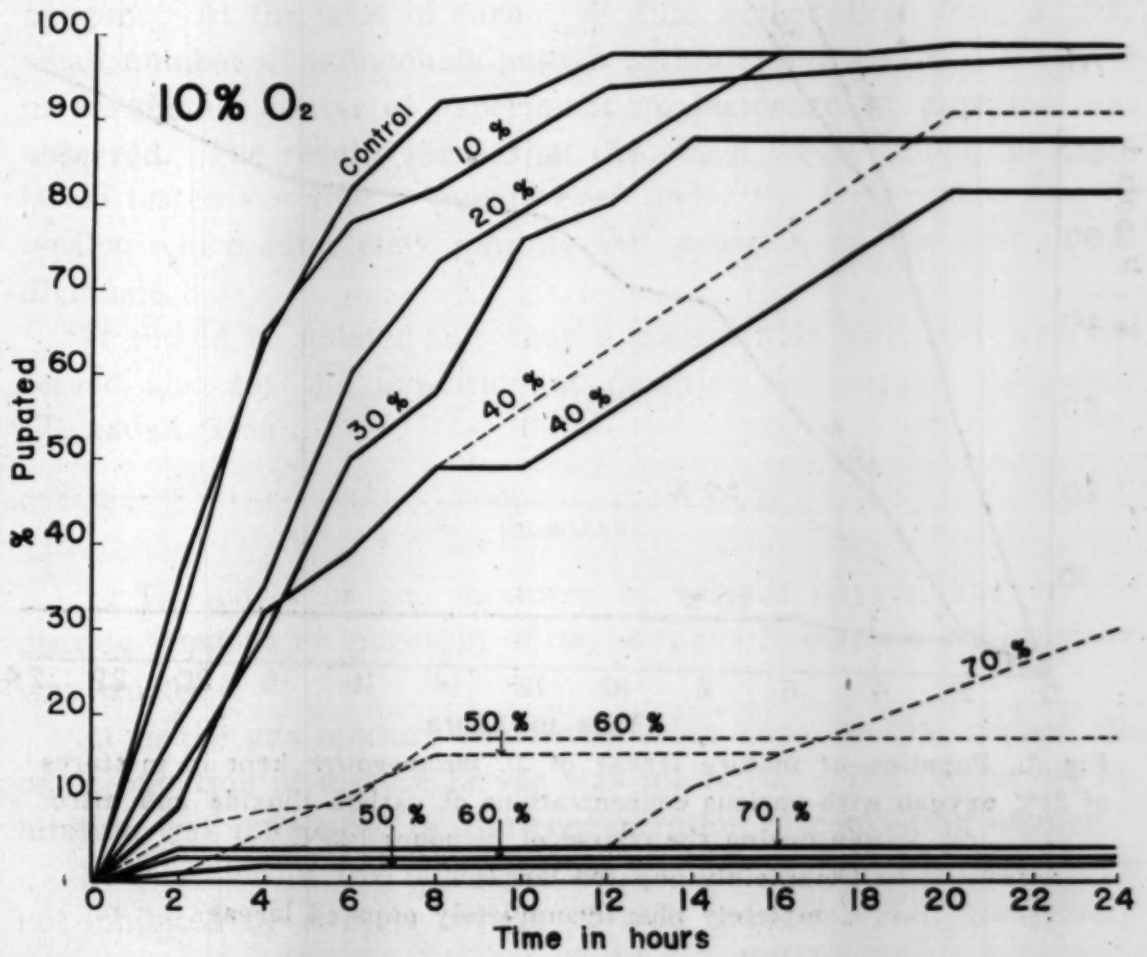

Fig. 2. Pupation of mature larvae of *D. melanogaster* kept in mixtures of 10% oxygen with various concentrations of carbon dioxide and nitrogen during the course of 24 hours (25°C.)
——: Completely pupated larvae
- - - -: Completely plus incompletely pupated larvae

C. Exposure to mixtures of 21% oxygen with 30, 40 and 50% carbon dioxide at 25°C. (Fig. 3)

In the mixtures containing 40% or more carbon dioxide, the significant suppression of pupation was observed. Especially in the series with 50% carbon dioxide, a low incidence of pupation was reached within 6 hours which remained unchanged later. No significant inhibition of pupation was caused by 30% carbon dioxide.

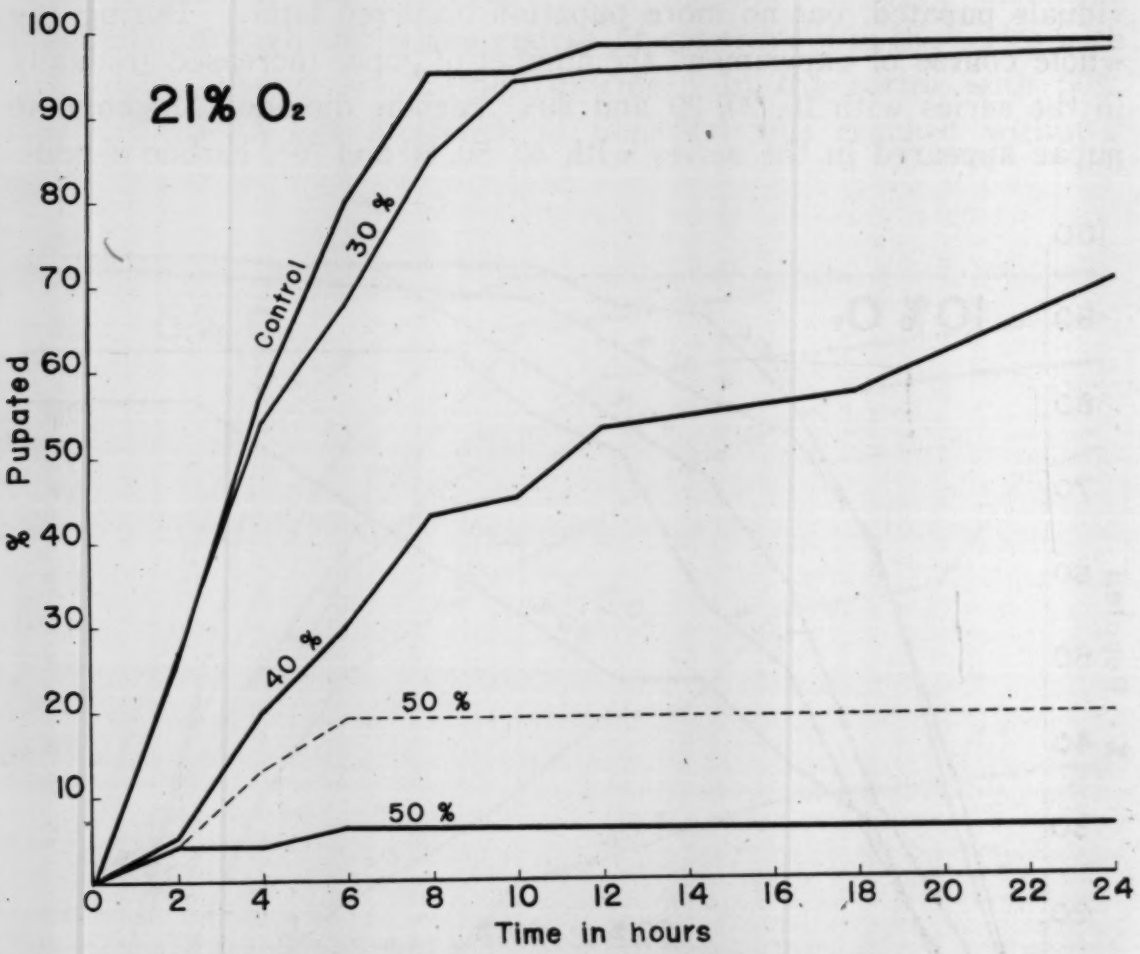

Fig. 3. Pupation of mature larvae of *D. melanogaster* kept in mixtures of 21% oxygen with various concentrations of carbon dioxide and nitrogen during the course of 24 hours (25°C.)
——: Completely pupated larvae
----: Completely plus incompletely pupated larvae

Conclusion

In the previous paper (Takaoka, 1959) an account was given of the effect of oxygen deficiency on pupation of the mature larvae of

Drosophila melanogaster. That carbon dioxide may also be a controlling factor of pupation is suggested by the life history of *Zeugodacus depressus* SHIRAKI. The larva of this species hatches and grows within the pumpkin fruit. Pupation occurs only after the larva leaves the fruit to migrate into the ground (TAKAMATSU, 1952). The exceptionally high carbon dioxide tension found by the present writer (unpublished) in the cavity of the pumpkin fruit may suppress the pupation of the larva while it is staying in the fruit.

In *Drosophila melanogaster*, experiments with gas mixtures containing various concentrations of carbon dioxide combined with oxygen indicate that in the mixture with carbon dioxide higher than 30–40%, pupation of mature larvae is strongly inhibited, and that the degree of inhibition is influenced by concentration of coexisting oxygen. At the level of carbon dioxide higher than 50% a very small number of individuals pupate within a few hours. However in the further course of experiment no incidence of pupation was observed. The results imply that the oldest larvae among the population tested are able to pupate even under the high carbon dioxide tension which completely inhibits the pupation of the rest of individuals.

It should be pointed out that a comparable situation was observed also for the inhibition of pupation by oxygen deficiency (TAKAOKA, 1959).

SUMMARY

1) The effect of gas mixtures of various oxygen and carbon dioxide tensions on pupation of the larvae of *Drosophila melanogaster* was studied.

2) In the gas mixture containing more than 30–40% carbon dioxide pupation of mature larvae was inhibited. The degree of inhibition was influenced by the concentration of coexisting oxygen.

3) Pupation of the oldest larvae of the population tested was not inhibited by a high carbon dioxide tension which completely suppressed pupation of the rest of the population.

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THE EFFECTS OF SULFHYDRYL REAGENTS ON ARTIFICIAL PARTHENOGENESIS IN THE SEA URCHIN EGG

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According to LOEB (1906), parthenogenetic development of the sea urchin egg fails to occur when a small amount of potassium cyanide is added to the hypertonic sea water which is used as the second agent in his method of double treatment. The author (1954, 1957) after confirming this fact further obtained similar results by using dinitrophenol and sodium azide instead of potassium cyanide. These results seem to imply that such chemicals make the hypertonic treatment ineffective for the induction of any parthenogenetic development because of a disturbance in the energy yielding phosphorus metabolism.

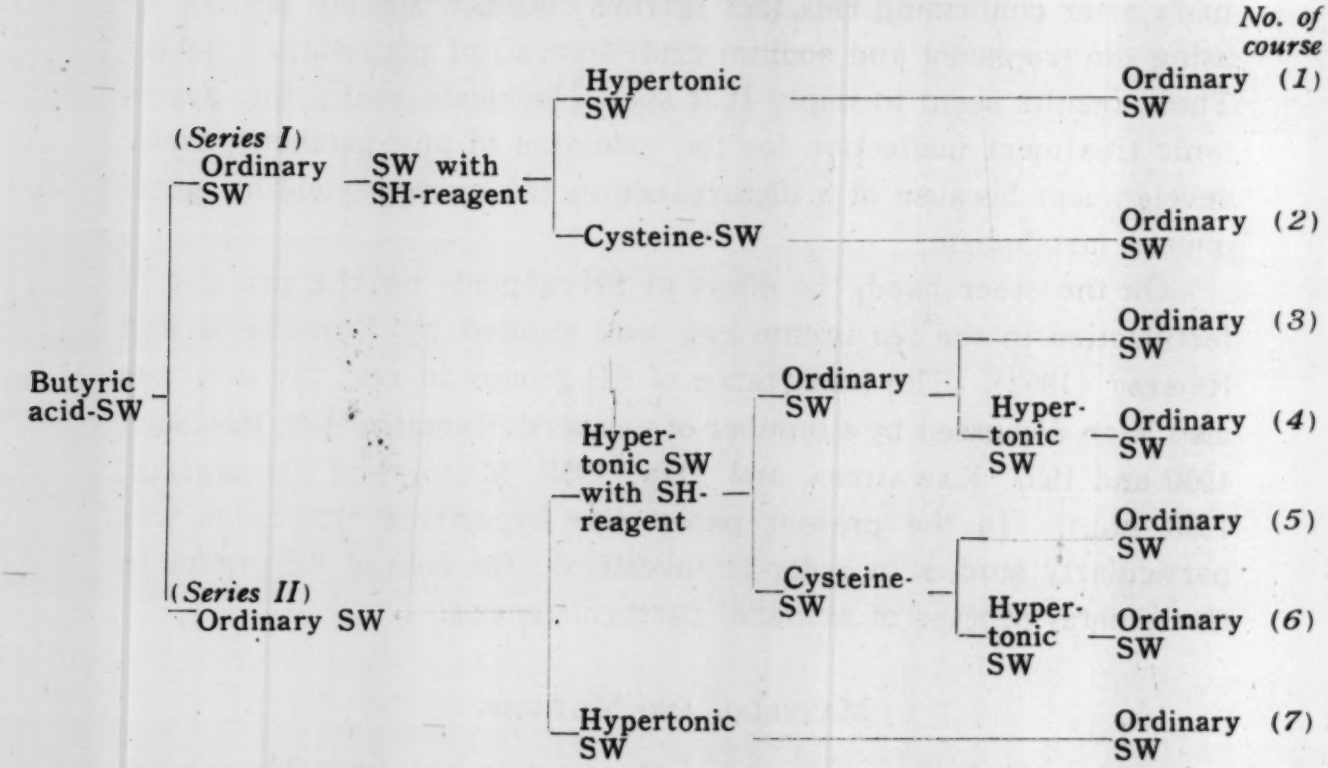
On the other hand, the effect of SH-reagents on the process of fertilization in the sea urchin egg was studied by RUNNSTRÖM and KRISZAT (1952). The importance of SH-groups in cell division has also been discussed by a number of workers (RAPKINE, 1931, BRACHET, 1950 and 1957, KAWAMURA and DAN, 1958, MAZIA and ZIMMERMAN, 1958, etc.). In the present paper, the hypertonic treatment was particularly studies in order to investigate the role of SH-groups in the general process of artificial parthenogenesis.

MATERIAL AND METHODS

The eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were emplyed as material. Unfertilized eggs were subjected to the double treatment of LOEB for artificial parthenogenesis (1913). The first treatment, by which the membrane formation is induced, was carried out through the use of 6 per cent butyric acid-sea water (6 cc. of N/10 butyric acid plus 94 cc. of sea water). The hypertonic sea water used for the second treatment, was prepared by adding 16 cc. of 2.5 N NaCl to 100 cc. of sea water.

The experiments were performed along the 7 courses shown in Table 1, using monoiodoacetamide (IAA), *p*-chloromercuribenzoate (PCMB) and mercuric chloride as SH-reagents. 0.1 M IAA, 2.8×10^{-3} M PCMB and 10^{-4} M $HgCl_2$ were prepared as stock solutions. PCMB was dissolved first in a small amount of weak alkaline solution, and then added to sea water, while IAA and $HgCl_2$ were dissolved directly in sea water. The stock solutions were diluted with sea water a short time before use. 10^{-2} M cysteine-sea water at pH 8.2 were used in order to test whether or not cysteine can reverse the effects of the SH-reagents.

Table 1. General experimental procedure to test the effects of the SH-reagents on the induction of cleavage by the double treatment of LOEB (SW; Sea water)



EXPERIMENTAL RESULTS

1. *Effect of IAA*

A group of experiments were performed along *Course 1* of Table 1. It was found that when activated eggs were exposed to 10^{-4} M IAA-sea water for 20 minutes, the induction of cleavage by the subsequent hypertonic treatment was suppressed though slightly.

As the length of time between the membrane formation and the hypertonic treatment was limited to 20 minutes, no further experiments could not be undertaken in *Series I.*

The next group of experiments were performed in accordance with *Series II* of Table 1. Eggs were activated by the butyric acid-treatment, and, 20 minutes after the membrane formation, they were put into hypertonic sea water with and without IAA. A part of the eggs which had been treated with hypertonic IAA-sea water were transferred to a 10^{-2} M cysteine-sea water while the rest were placed in ordinary sea water. As shown in Table 2, the eggs treated with the hypertonic sea water without IAA showed a high percentage of cleavage, while the eggs treated with the hypertonic sea water containing IAA showed no cleavage even after being exposed to cysteine-sea water for 20 minutes (Fig. 1 a). When such eggs were again treated with hypertonic sea water which did not contain IAA, their cleavage was induced (Fig. 1 b).

Table 2. Effects of the addition of IAA to hypertonic sea water on the percentage of parthenogenetic cleavage

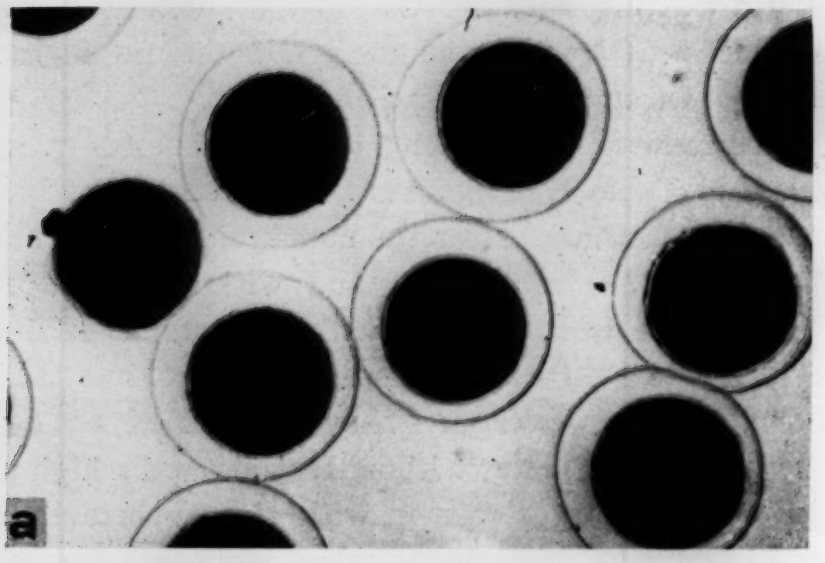
Temp. 18°C.

Time-length of 1st. hypertonic treatment (min.)			30	40	50
Eggs activated by butyric acid treatment and, after 20 min., treated with hypertonic *IAA*-sea water	placed in ordinary sea water	No. of *course* (*3*)	0%	0%	0%
	followed by second hypertonic treatment for 30 min.	(*4*)	0	0	0
	exposed to cysteine-sea water	(*5*)	0	0	0
	exposed to cysteine-sea water, followed by second hypertonic treatment for 30 min.	(*6*)	22	36	38
Eggs activated by butyric acid treatment and, after 20 min., treated with *ordinary* hypertonic sea water		(*7*)	45	62	52

Activation by butyric acid with a subsequent hypertonic treatment containing *IAA* (10^{-4} M) and that followed by cysteine (10^{-2} M) and the second hypertonic treatment are compared.

2. Effect of PCMB

Similar experiments were carried out substituting PCMB for IAA. The concentration of the PCMB solution used was 7×10^{-4} M.



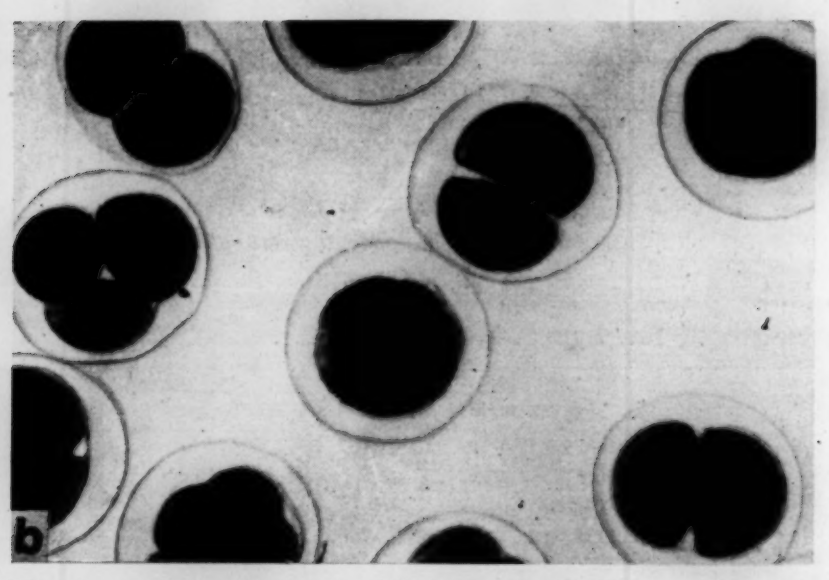


Fig. 1 a, Eggs treated with hypertonic IAA-sea water (*Course 3*). Cleavage failed to occur.——b, Eggs treated with hypertonic IAA-sea water, followed by cysteine treatment and the ordinary hypertonic treatment (*Course 6*). Cleavage was induced.

In *Series I*, activated eggs were exposed to the PCMB solution immediately after the membrane formation. These eggs showed no cleavage if they were treated only with hypertonic sea water, but they became capable of cleavage if they were washed with a 10^{-2} M cysteine-sea water for 20 minutes and then were treated with hypertonic sea water. In *Series* II, activated eggs were treated with hypertonic PCMB-sea water. In these eggs no cleavage was induced even

after being washed in cysteine-sea water (*Course 5*). These eggs, however, showed cleavage, if they were subjected to the second hypertonic treatment without PCMB (*Course 6*). One of these results is shown in Table 3.

From these results it might be said that hypertonic treatment becomes ineffective for the induction of cleavage, when SH-groups in the egg are blocked by IAA or PCMB, and after this blook is released by cysteine, it becomes effective again.

Table 3. Effects of PCMB on the percentage of parthenogenetic cleavage

Temp. 19°C.

Time-length of 1st. hypertonic treatment (min.)			20	30	40
Eggs treated with PCMB immediately after membrane formation, and then exposed to hypertonic sea water		No. of *course* (*1*)	0%	0%	0%
Eggs treated with PCMB as above, and then exposed to cysteine-sea water, followed by hypertonic sea water		(*2*)	35	53	64
Eggs activated by butyric acid treatment and, after 20 min., treated with hypertonic *PCMB*-sea water	placed in ordinary sea water	(*3*)	0	0	0
	followed by second hypertonic treatment for 30 min.	(*4*)	0	0	0
	exposed to cysteine-sea water	(*5*)	0	0	0
	exposed to cysteine-sea water, followed by second hypertonic treatment for 30 min.	(*6*)	10	25	43
Eggs activated by butyric acid treatment and, after 20 min., treated with *ordinary* hypertonic sea water		(*7*)	8	54	78

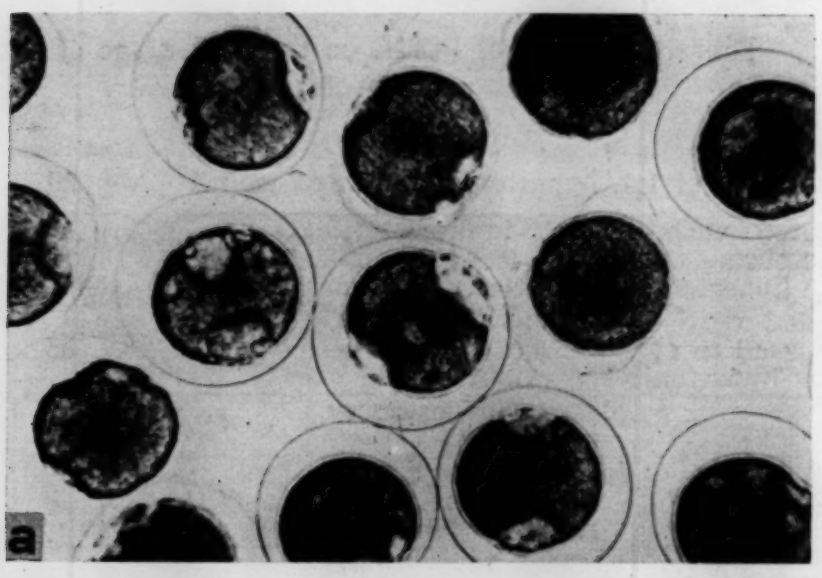
Activated eggs were exposed to *PCMB* (7×10^{-4} M) either before or during the hypertonic treatment. The treatment with cysteine (10^{-2} M) and second hypertonic treatment were performed in accordance with Table 1.

3. Effects of $HgCl_2$

Experiments similar to the foregoing were performed using $HgCl_2$ (10^{-5} M). No cleavage was induced in *Course 1*. In some cases, cleavage proceeded only halfway in the furrowing process. It was noticed that the hyaline layer became swollen in the $HgCl_2$-sea water, and the egg surface began to roughen after the eggs

were returned to ordinary sea water. This roughening, however, did not occur when the eggs had been washed with cysteine-sea water. Such eggs showed cleavage, though in a low percentage, if they were subjected to an appropriate treatment with hypertonic sea water (*Course 2*).

In *Courses 3* and *4*, most of the eggs failed to cleave, although some eggs showed an incomplete furrow formation (Fig. 2 a), while



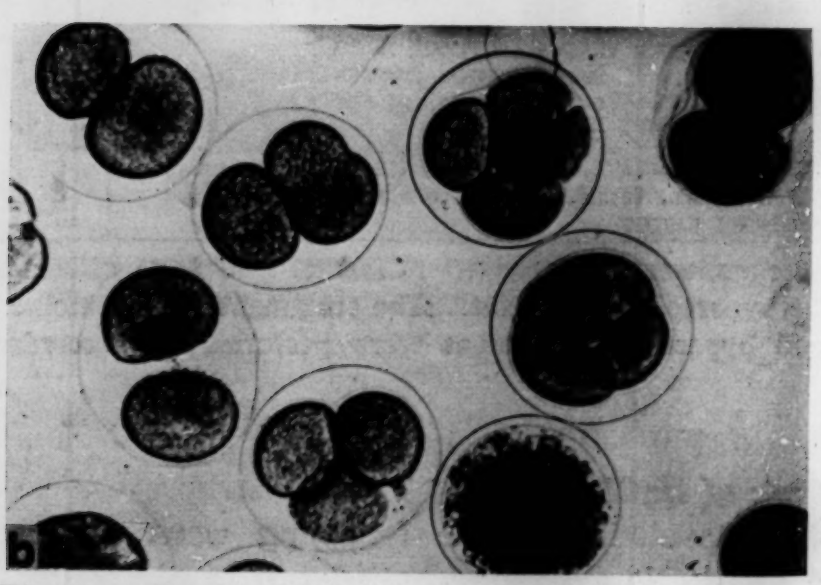


Fig. 2. a, Eggs treated with hypertonic $HgCl_2$-sea water (*Course 3*). Cleavage did not occur.——b, Eggs treated with hypertonic $HgCl_2$-sea water and then washed with cysteine-sea water (*Course 5*). Cleavage occurred.

in *Courses 5* and *6*, cleavage took place after they were put into cysteine-sea water (Fig. 2 b). One of these results is shown in Table 4.

Table 4. Effects of $HgCl_2$ on the percentage of parthenogenetic cleavage

Temp. 19°C.

Time-length of 1st. hypertonic treatment (min.)			20	30	40
Eggs treated with $HgCl_2$ immediately after membrane formation, and then exposed to hypertonic sea water		No. of *course* (*1*)	0%	0%	0%
Eggs treated with $HgCl_2$ as above, and then exposed to cysteine-sea water, followed by hypertonic sea water		(*2*)	0	22	38
Eggs activated by butyric acid treatment and, after 20 min., treated with hypertonic *$HgCl_2$* sea water	placed in ordinary sea water	(*3*)	0	0	0
	followed by second hypertonic treatment for 30 min.	(*4*)	0	0	0
	exposed to cysteine-sea water	(*5*)	0	62	42
	exposed to cysteine-sea water, followed by second hypertonic treatment for 30 min.	(*6*)	68	65	72
Eggs activated by butyric acid treatment and, after 20 min., treated with *ordinary* hypertonic sea water		(*7*)	25	48	70

Activated eggs were exposed to $HgCl_2$ (10^{-5} M) either before or during the hypertonic treatment. The treatment with cysteine (10^{-2} M) and second hypertonic treatment were performed in accordance with Table 1.

Discussion

The SH-groups are supposed to play important roles in physiological and morphological processes of cell division. Rapkine (1931) demonstrated that glutathione in the sea urchin egg decreases to a minimum at the monaster stage and then increases to a maximum just before the first cleavage. Mazia and Dan (1952) showed that the chemical composition of the isolated mitotic apparatus is closely related to protein-bound SH-groups. Kawamura and Dan (1958) recently studied the distribution of protein-bound SH-groups during cleavage, and found that the astral centers and spindle during mitosis can be deeply stained with SH-specific dye. They assumed that the

SH-protein occurring in the centrosphere is the precursor material for the aster and spindle. SAKAI (1958) reported that contractile fibrous protein can be extracted from sea urchin eggs and that the amounts of the SH-groups in this protein fluctuate during a division cycle. More recently, SAKAI and DAN (1959) have reported on the amounts of reduced and oxydized glutathione during early cell division.

In the present study it has been found that the artificial induction of cleavage by the hypertonic treatment is suppressed, when the SH-reagent, such as IAA- and PCMB, has been added to the hypertonic sea water. The IAA- and PCMB-treated eggs, however, begin to cleave, if hypertonic treatment devoid of SH-reagents is given after the eggs have been washed with cysteine-sea water. These results suggest the possibility that the SH-groups which participate in the composition of the mitotic apparatus may be activated during the hypertonic treatment.

When activated eggs are treated with hypertonic sea water containing $HgCl_2$, although no cleavage occurs as in the cases of IAA and PCMB, there is a difference in the reversibility of the effect between the two cases. The $HgCl_2$-treated eggs cleave on washing with cysteine-sea water without subsequent hypertonic treatment.

From this fact, it may be assumed that $HgCl_2$ affects the mechanism of the furrowing rather than the mechanism of the initiation of cleavage. According to NICKERSON and FALCONE (1956), mitochondrial particulates isolated from the divisionless mutant of yeast completely lack in ability to reduce -SS- of the cell-wall protein of a normal strain although the normal strain exhibits vigorous protein disulfide reductase activity against the cell-wall protein obtained from this strain and from the mutant. The reaction in which -SS- in the cell-wall protein is reduced to SH- is considered important in cell division of the microoganism. These findings may harmonize with the above-mentioned assumption.

In 1957, the author suggested that the cleavage-inducing effect of the hypertonic medium in parthenogenesis may be partly associated with a process involving oxidative phosphorylation. It may also be considered that the induction of cleavage by the hypertonic treatment is closely associated with the SH-groups.

Summary

1. The effects of SH-reagents on the induction of cleavage by the double treatment of LOEB were studied, using *Hemicentrotus* eggs as material.

2. When activated eggs were exposed to PCMB or $HgCl_2$ immediately after the membrane formation, they became incapable of being induced to cleave by the hypertonic treatment. These eggs, however, recovered from such a state, when they were washed with cysteine-sea water.

3. When the SH-reagents were added to the hypertonic sea water, the induction of cleavage failed to occur. This effect was reversed when the eggs were washed with cysteine-sea water. In the $HgCl_2$-treated eggs, cleavage took place after the cysteine treatment, while in the PCMB- or IAA-treated eggs, a second hypertonic treatment had to be used again to induce cleavage.

4. It is suggested that the indution of cleavage by the hypertonic treatment, is closely associated with the SH-groups.

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STUDIES ON THE PHYSIOLOGY OF *ARTEMIA* EMBRYOS

I. RESPIRATION AND ITS MAIN SUBSTRATE DURING THE EARLY DEVELOPMENT OF THE ENCYSTED EMBRYO*

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INTRODUCTION

The branchiopod crustacean *Artemia salina* LEACH, as is well known, lives in salt lakes or brine pools of considerably high salinities in most parts of the world (DADAY DE DEÉS, 1910; ABONYI, 1915; MARTIN and WILBUR, 1921; RELYEA, 1937; etc.). It reproduces by forming either viviparous youngs or encysted embryos surrounded by the thick shell. The latters are formed in the dry season, and rest until they come in salt water of adequate salinity to hatch (WHITAKER, 1940).

The encysted embryos are multinucleate, but they do not develop into blastulae. The development seems to halt before desiccation of embryos. Under the laboratory condition, they remain viable for a good many years (WHITAKER, 1940; DEMPSTER and HANNA, 1956) and when incubated in salt water of an appropriate salinity at a suitable temperature, they take up water and hatch as nauplii in one day or more.

Maintenance of viability of *Artemia* embryos under desiccation and their resumption of development by water infiltration offer interesting problems in the field of physiology.

As to the metabolism related with the respiration of the embryos, investigations have scarecely been carried out hitherto. Recently, however, URBANI (1959) reported on the successive changes

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of the amounts of proteins, carbohydrates and lipids during the development.

The present paper deals with results of the investigations concerning the endogenous respiration of the embryos in the periods, both resting and developing, with special references to substances used as the substrate.

Material and Methods

The encysted embryos of the brine shrimp provided by San Francisco Aquarium Society were used as material in the present work. They were collected in July 1956 and the experiments were carried out from October 1957 to February 1958.

According to Whitaker (1940), there is an ambiguity in the taxonomy of *Artemia* species, and the Californian brine shrimp has been regarded either as a variety of *A. salina* or as a distinct species, *franciscana*. Pennak (1953), however, maintained a view that there is in the genus *Artemia* only one cosmopolitan species, *salina*, which has numerous intergrading physiological and morphological varieties. The present author, therefore, assumes his material to be *A. salina* for convenience' sake.

The medium in which the dry embryos were suspended for development was 2% NaCl solution buffered with M/15 phosphate at pH 7.3–7.5 and kept at 27°C. Under this condition, about 70% of the embryos were able to hatch.

O_2 uptake and CO_2 output were measured manometrically according to Warburg's direct method (Umbreit *et al.*, 1949). In CO_2 output estimation, retained CO_2 in the medium was liberated to gas phase by an addition of 3 N H_2SO_4.

For detection and identification of sugars, paper-chromatography (Toyo filter paper No. 50, strip shape of 2×40 cm, by ascending technique) was employed. As the developing solvents, upper layers both of butanol—acetic acid—water mixture (4:1:5) and of ethyl acetate—acetic acid—water mixture (3:1:3) were used. Spots of reducing sugars on the chromatogram were detected by spraying ammoniacal $AgNO_3$, *i.e.* a mixture of equal volume of 0.1 N $AgNO_3$ and 5 N NH_4OH (Partridge and Westall, 1948). The embryos were homogenized with water in an ice bath. This homogenate was

divided into two parts, one of which was hydrolyzed with H_2SO_4 at the final concentration of 1 N in a boiling water bath for about four hours. The hydrolyzate was centrifuged and the supernatant was neutralized with 1 N Na_2CO_3. The detection of sugars was carried out both on hydrolyzed and on unhydrolyzed samples. For deproteinization (SOMOGYI, 1945 b), two volumes of 0.3 N $Ba(OH)_2$ were mixed with one volume of the supernatant, to which were then added two volumes of 5% $ZnSO_4 \cdot 7\,H_2O$ and this mixture was centrifuged. The supernatant was dried at 100°C., and the residue was dissolved in dry pyridine at 100°C. for ten minutes, then cooled and filtered to desalt. Pyridine in the filtrate was removed by distillation under reduced pressure at room temperature (MALPRESS and MORRISON, 1949). These concentrated sugar solutions were applied to filter paper. Glucose (Merck and Co.) was used as standard to make comparison of the Rf value.

Glycogen was measured by the colorimetric method using anthrone reagent (1% anthrone in ethyl acetate) (KAHAN, 1953). To obtain the developing embryos, the dry embryos suspended in the medium were incubated for a certain period of time, and collected by straining with silk cloth. The embryos were homogenized with 5% trichloroacetic acid and centrifuged. Thus obtained supernatants were employed as samples. Intensity of the colour developed by the anthrone reaction was measured with the spectrophotometer (Hitachi EPU-2) at 625 mμ. Glycogen (Merck and Co.) was used as the standard.

The amount of reducing sugars was also determined by colorimetric method. The embryos were homogenized with water in an ice bath. Immediately after centrifugation, supernatants of the homogenates were deproteinized by adding $Ba(OH)_2$ and $ZnSO_4$. Reducing sugars extracted in the supernatant were determined by the method of SOMOGYI (1945 a, 1952) using copper reagent and arsenomolybdate solution. Intensity of the colour was measured at the maximal absorption of 750 mμ. Glucose (Merck and Co.) was used as the standard.

RESULTS

Respiration rate and respiratory quotient (*R.Q.*). In Table 1

and Fig. 1, respiration rate (Qo_2* and Qco_2**) and R.Q.*** of embryos both in resting and at various periods of early development are shown.

The respiration rate of resting embryos was measured without addition of water. However, the volume of consumed O_2 and that of released CO_2 of dry embryos were so small that respiration was considered to be negligible as compared with that of developing ones which will be given below.

In order to measure the respiration rate of embryos at various periods of development, dry embryos were suspended in salt water in flasks of WARBURG manometer and were incubated at 27°C for 2, 5, 8, and 12 hours respectively. To avoid the undue decrease of oxygen in the medium, the flasks were occasionally shaken. When a desired stage of development was reached, O_2 uptake and CO_2 output were measured for two hours, and Qo_2 and Qco_2 for the two hours' period were considered to represent those at the end of the first hour.

The values of Qo_2 and Qco_2 given in Table 1 are means of four measurements and their standard errors. The mean R.Q. is the ratio of the mean Qco_2 to the mean Qo_2, the maximum of R. Q. is the ratio of the maximal Qco_2 to the minimal Qo_2, and the minimum is *vice versa*.

As read in the table, there was a tendency of increase both in Qo_2 and in Qco_2 as the development proceeded, with an exception

Table 1. Qo_2, Qco_2 and R.Q. of *Artemia* embryos at various periods of development

Incubation time in hours	Qo_2	Qco_2	R.Q.		
			Mean	Maximum	Minimum
0(Resting)	0	0	—	—	—
1	0.95±0.02	0.85±0.00	0.90	0.91	0.88
3	1.14±0.05	1.02±0.01	0.90	0.95	0.85
6	1.00±0.04	0.93±0.05	0.93	1.02	0.85
9	1.32±0.03	1.21±0.04	0.92	0.97	0.87
13	1.45±0.06	1.34±0.05	0.93	1.00	0.86

* μl of O_2 consumed per mg of dry weight per hour.
** μl of CO_2 given off per mg of dry weight per hour.
*** calculated as Qco_2/Qo_2.

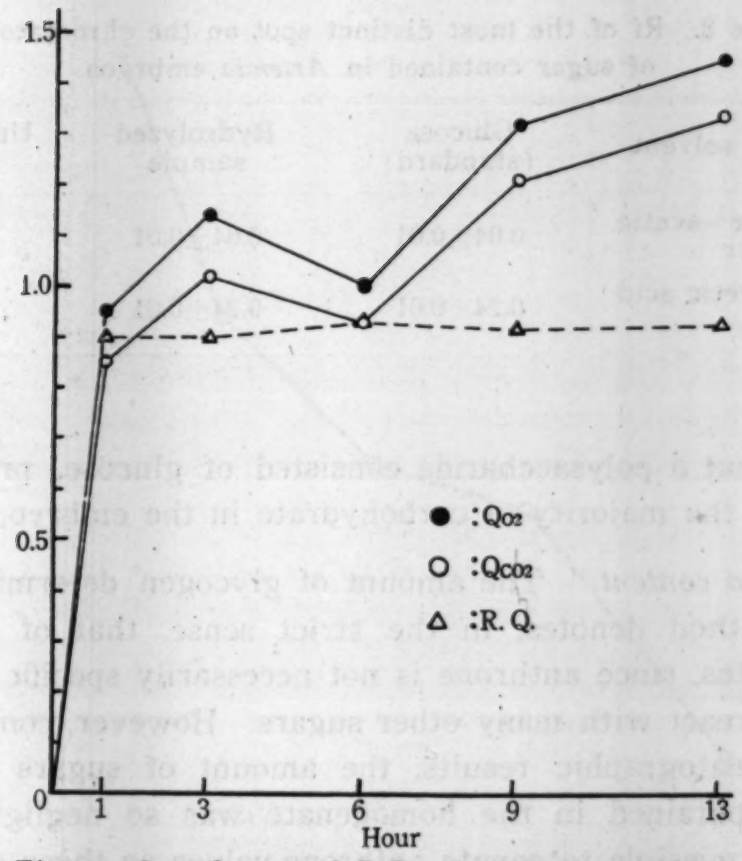

Fig. 1. Q_{O_2}, Q_{CO_2} and R.Q. of *Artemia* embryos at various periods of development (See Table 1). Abscissa: Incubation time in hours, Ordinate: Quantity.

of a temporary decrease at about the 6th hour of incubation. The mean R.Q. remained nearly constant at 0.9 or slightly higher throughout the period, at least up to 12th hour of incubation.

Detection and identification of sugars by paper-chromatography. In the present experiment, both hydrolyzed and unhydrolyzed homogenate of the resting embryos were used as material.

Both in hydrolyzed and in unhydrolyzed samples, spots which could be developed by ammoniacal $AgNO_3$ were only a few in number and most of them were very faint, so that it was difficult to identify them even qualitatively. This was particularly so in the unhydrolyzed sample (Table 2). In spite of it, it was conspicuous that the only distinct spot occurred in hydrolyzed sample which was missing in unhydrolyzed sample. This spot was identified as glucose (see Rf value of Table 2).

From the size and intensity of the colour of this spot, it was

Table 2. Rf of the most distinct spot on the chromatogram of sugar contained in *Artemia* embryos

Developing solvent	Glucose (standard)	Hydrolyzed sample	Unhydrolyzed sample
Ethyl acetate—acetic acid—water	0.04±0.01	0.04±0.01	—
Butanol—acetic acid—water	0.24±0.01	0.24±0.01	—

surmized that a polysaccharide consisted of glucose, probably glycogen, was the majority of carbohydrate in the embryo.

Glycogen content. The amount of glycogen determined by the present method denotes, in the strict sense, that of acid-soluble carbohydrates, since anthrone is not necessarily specific to glycogen but it can react with many other sugars. However, considering the paper-chromatographic results, the amount of sugars other than glycogen contained in the homogenate was so negligible that it may be permissible to equate anthrone-values as those of glycogen.

The content of glycogen in the embryos, both in resting and in developing period, is shown in Table 3 and Fig. 2. The values given are means of two measurements.

The amount of glycogen in the resting embryos was about 18% of their dry weight. Glycogen was markedly consumed during the first two hours of incubation in salt water. In the following six hours, the content of glycogen decreased slowly, but rather rapidly thereafter. At the end of the 12th hour, the glycogen content was reduced to about 11% of the embryo dry weight. The glycogen consumed during 12 hours of development was about 70 μg per mg dry weight of the embryo or about 38% of glycogen content of the resting embryos.

Table 3. Glycogen content of *Artemia* embryos at various periods of development

Incubation time in hours	0(Resting)	2	5	8	12
Glycogen content, μg/mg of dry weight	182.6±2.9	156.4±4.9	141.8±5.0	137.5±8.0	113.1±9.7

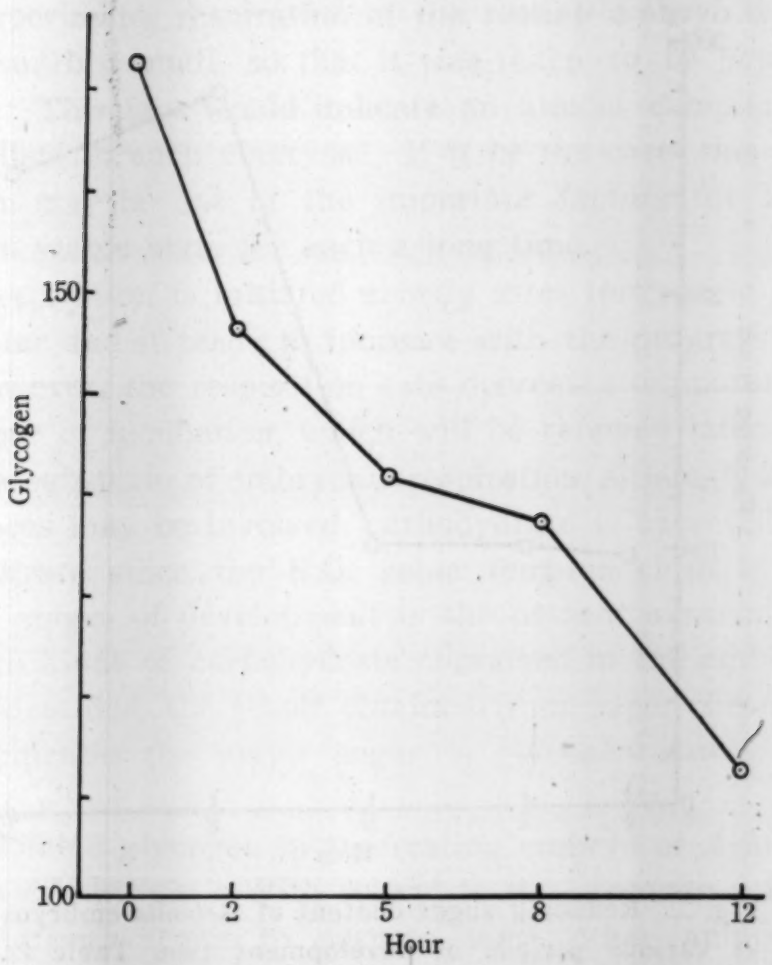

Fig. 2. Glycogen content of *Artemia* embryos at various periods of development (see Table 3). Abscissa: Incubation time in hours, Ordinate: Glycogen amount, μg/mg of dry weight of embryos.

Reducing sugar content. Table 4 and Fig. 3 show the amount of reducing sugars contained both in resting and in developing embryos. It was determined by SOMOGYI's method and expressed in terms of glucose amount. The values given are means of four measurements.

The content of reducing sugars in the embryo was nearly 0.1%

Table 4. Reducing sugar content of *Artemia* embryos at various periods of development

Incubation time in hours	0(Resting)	2	5	8	12
Reducing sugar content, μg/100 mg of dry weight	97.1±5.9	101.0±5.4	102.4±4.5	281.7±16.1	250.6±11.3

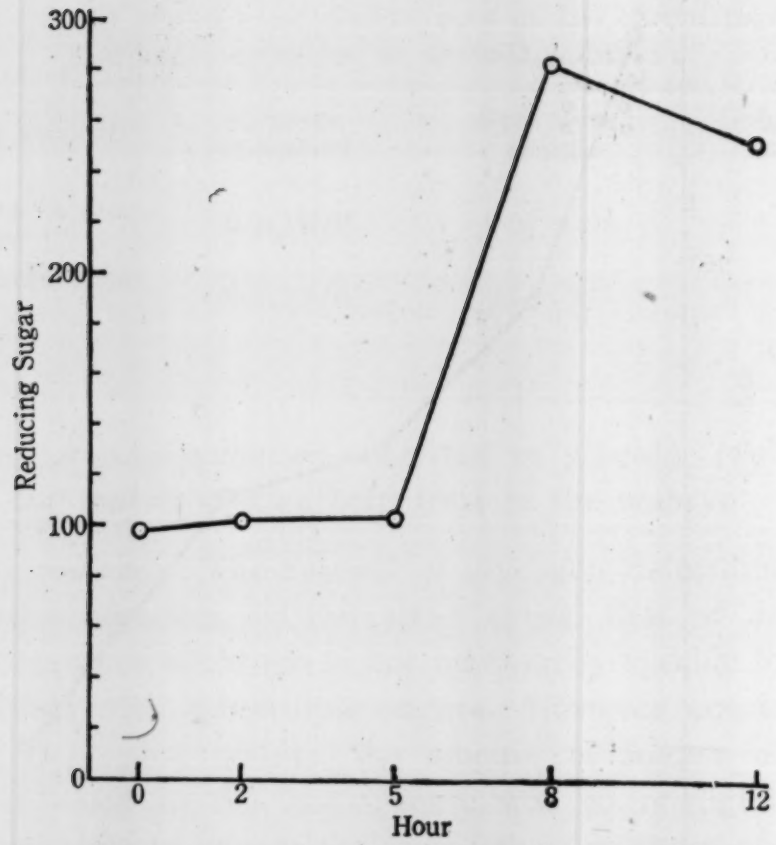

Fig. 3. Reducing sugar content of *Artemia* embryos at various periods of development (see Table 4). Abscissa: Incubation time in hours, Ordinate: Amount of reducing sugars, μg/100 mg of dry weight of embryos.

of its dry weight in the resting state, and after resumption of development, it remained almost unchanged at least up to the end of the 5th hour. Between the 5th and the 8th hour, however, it markedly increased to 0.28%, then slightly decreased to 0.25% in the following four hours.

In comparison with glycogen, reducing sugars were much less in quantity, corresponding nearly to 0.5 to 2.0% of the glycogen content determined in the foregoing experiment.

Discussion

Since the encysted dry embryos of *Artemia* remain viable for many years in the resting state, it may be worthy to investigate to what extent the metabolism is going on in such embryos. In the

present experiment, respiration of the resting embryo was found to be immeasurably small, so that it was taken to be practically nil (Table 1). This fact would indicate an almost complete cessation of metabolism in such embryos. If it be the case, this blocking of metabolism may be one of the important factors for keeping the embryos in viable state for such a long time.

The respiration is initiated shortly after immersing the embryo in salt water and it tends to increase with the progress of development. However, the respiration rate decreases temporarily at about the 6th hour of incubation, which will be referred later.

As the substrate of embryonic respiration, although several kinds of substances may be involved, carbohydrate is expected to be the main substrate since the R.Q. value remains close to 1 through successive stages of development in the present experiment.

Though kinds of carbohydrate contained in the embryo are not yet fully identified, the result obtained from paper-chromatographic analysis indicates the major sugar is glycogen stored in a large quantity.

Acid soluble glycogen in the resting embryo of *Artemia* reaches 18% of its dry weight. With regard to the glycogen content of the embryo, *Artemia* seems to surpass some other animals; *e.g.* in *Arbacia punctulata* the acid hydrolyzable carbohydrate is 7% of the egg dry weight (HUTCHENS *et al.*, 1942), in *Echinus esculentus* glycogen is 1.5% (CLELAND and ROTHCHILD, 1952), and in *Rana pipiens* total glycogen, of which 10% or less is acid insoluble, is 2% or less (FITCH and MERRICK, 1958). Therefore, the glycogen content of the resting embryo of *Artemia* is about 10 times higher than that of *Echinus* and of *Rana* and exceeds that of *Arbacia* by 2.5 times. According to URBANI (1959), the carbohydrate in the resting embryo of *Artemia* is 22.6%, and it gradually decreases when the embryo resumes its development. These facts seem to agree with the present results essentially.

About 38% of the initial glycogen are consumed during 12 hours of development. The comparison of Fig. 1 with Fig. 2 indicates that there seems to exist an approximate parallellism between the change in respiration rate and that in the rate of glycogen consumption at least in the early period of development. As stated before, there is a temporary decrease of the respiration rate at about

the 6th hour of incubation. This conforms to temporary slowing down of glycogen consumption between the 5th and the 8th hour. In consequence of such simultaneous decrease in respiration and glycogen consumption, the energy production by means of oxidation of glycogen is expected to decrease. Although what this temporary change in energy production means for the developmental mechanism might be explained in several ways, more morphological evidences of the developmental processes are needed to approach the problem.

Notwithstanding such an approximate parallelism, the amount of glycogen consumed during the early development is notably larger than can be accounted for by the combustion of it. Both the amount of O_2 taken up and that of CO_2 released by the embryo are about ten times smaller than expected from complete oxidation of glycogen. Therefore, it may be supposed that a considerable amount of the glycogen is not oxidized completely but remains as intermediates of glycolysis or is transformed into some other substances which may be accumulated in the embryo.

Reducing sugars present in the embryo is so small in quantity in comparison with both glycogen content and its consumption during development that they cannot be either the main substrate of respiration or the principal substance accumulated as an intermediate. The total amount of reducing sugars increases rapidly from 0.1% to 0.3% of the dry weight of the embryo in a period between the 5th and the 8th hour of incubation. This period of development corresponds to the time of decrease both in the rate of respiration and in that of glycogen consumption. So the increase of reducing sugars might have some connection with glycolysis.

A similar series of experiments was performed using the encysted embryos of *Artemia* from Great Salt Lake in Utah as material. Results obtained were almost the same as those in the present work.

The author wishes to express his deep gratitude to Prof. K. NAKAMURA for his kind guidance and advice. Thanks are also due to Dr. M. RAKOWICZ of San Francisco Aquarium Society for his generosity in providing the material.

SUMMARY

1. The respiration and its substrate of embryos of *Artemia*

salina from California were studied both in the resting and in the developing period.

2. Respiration was practically nil in the resting embryo, but was initiated shortly after immersing the embryos into salt water. It increased thereafter as the development proceeded with an exception of a temporary decrease at about the 6th hour of incubation.

3. R.Q. remained between 0.90 and 0.93 through the development.

4. Glucose was detected by paper-chromatography in the hydrolyzate of the embryo homogenate, though it is hardly discernible in the unhydrolyzed sample. Other reducing sugars could not be identified.

5. Glycogen was about 18% of the dry weight of the resting embryos. It decreased to about 15.5% in the first two hours of incubation, and to about 11% at the end of the 12th hour.

6. Content of reducing sugars in the embryo was about 0.1% of its dry weight at the resting stage. It remained unchanged up to the 5th hour of incubation, and increased rapidly to about 0.28% in the following three hours.

7. The relation between the rate of respiration and that of carbohydrate consumption was discussed.

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ANALYSIS OF GENE ACTION IN THE TRANSPARENT-SCALED GOLDFISH, *CARASSIUS AURATUS*

I. ON THE GENE ACTION IN THE DISAPPEARANCE OF GUANOPHORES

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INTRODUCTION

The scale transparency, namely the lack of reflecting guanine layers in scales and dermis, is one of the rare mendelian characters of the goldfish which have been exactly proved by genetic analysis. This character was at first termed "hypolepidose" (*Hy Hy*) by BERNDT ('25) and was considered to be dominant to the normal (*hy hy*). But shortly after it, CHEN ('28) demonstrated that the dominance of the factor is imperfect. He distinguished homozygous and heterozygous types in the transparent-scaled fish, applying symbols *T'* and *T* for the transparent and the normal scale-character respectively. The heterozygous fish (*TT'*) has both transparent and normal scales in mosaic patterns, so that the fish appears mottled in colour.

MATSUI ('33, '34) found another type of the scale transparency, being recessive to the normal-scaled and hypostatic to the usual transparent character, which he called "net-like transparent" (*n*; normal scale=*N*). He demonstrated that these types of scale transparency are inherited as mendelian dihybrid, and described their genetical constitutions as follows: All transparent (*TN* or *Tn*), mosaic transparent (*TtN*), net-like transparent (*tn*) and normal character (*tN*). Since the present investigation does not consider the net-like transparent-scaled type, the following designation was adopted: Homozygous transparent type (*TT*), heterozygous transparent type (*Tt*) and normal type (*tt*).

The normal type (*tt*) is represented by the ordinary goldfish.

The fish possesses reflecting layers of guanophores almost on the entire body surface, particularly abundant on the ventral surface and on the peritonium. The fish has also two kinds of chromatophores, that is, melanophores and xanthophores, which give rise to the characteristic colour patterns of the goldfish. They are usually most abundant on the dorsal side, and decrease towards the ventral side, where they are generally absent.

The fish of the homozygous transparent type (*TT*) differs markedly from the normal type. In this type not only the guanine layers on scales and dermis but also melanophores and xanthophores are usually lacking entirely on the body surface. Thus the presence of *T* in duplex condition usually causes complete loss of the three kinds of chromatophores and the fish seems perfectly pale in tint. In some fish of this type, however, certain areas on the head or/and at the anterior end of the caudal fin are pigmented with melanophores. Apart from the exceptional melanophore-pigmentation, there are also some cases in which yellow or red chromatophores develop in limited areas on the side of the trunk or head at the late larval stage.

The heterozygous type (*Tt*) is generally more complicated and variable in the expression of both pigmentation and reflecting guanine layers. The reflecting tissue is reduced to about two-thirds of the trunk surface. Melanophores and xanthophores are always mixed in the characteristic mottled condition. There are found more colour-spots on the dorsal and dorsolateral parts of the body than on the ventral part.

Goodrich and Hansen ('31) have studied the development of these colour characters in the offspring of the "shubunkin" (heterozygous transparent type). They found differences of the melanophore density among F_1-individuals in the early embryonic stage. In the homozygous transparent type a large number of melanophores began to degenerate, while the multiplication of it gradually slowed down, resulting a fading of the colour. In the normal type, however, melanophores increased their number steadily, without degeneration, until the larva has reached the stage of natural decolouration, which usually occurs more than two to three months after hatching. The heterozygous fish behaved intermediately between the two homozygous types, and showed wide variations of colour pattern. They

concluded from these observations that the genic factors apparently began to exert their influences in the early embryonic stage.

GOODRICH and HANSEN also observed the ontogenic development of reflecting guanine layers in the homozygous transparent type, and found that the layers are formed very irregularly and unsymmetrically on the body surface with wide variation in pattern. They further found that the reflecting layer of the eye is entirely lacking in this type, although it is completely formed in the normal type before hatching. From these observations they concluded that the genic influence upon the reflecting tissue of the eye became visibly effective during the pre-hatching period.

The first part of the present paper deals with a comparative study of the development of chromatophores, particularly of the reflecting guanine layers in the three genetical types mentioned above. It has been shown that although there is little difference in the mode of the formation of guanine layers among the three types in their early stage, the layers in the transparent type begin to degenerate almost all over the body surface around ten days after hatching. At the same time melanophores and xanthophores also begin depigmentation.

In its latter part, the present paper reports on transplantation and implantation of embryonic and larval eyes in various combinations between two different homozygous types, which are expected to clarify some problems of the mechanism of degeneration of the reflecting layers.

MATERIALS AND GENERAL METHODS

Materials used for experiments were in-bred homozygous transparent (*TT*) and homozygous normal (*tt*) types. The homozygous fish had been segregated by self-breeding of the heterozygous transparent-scaled fish "sanshoku-demekin" (*Tt*) (mottled telescope-eyed goldfish, CHEN '28; nacreous telescope-eyed goldfish, AFFLECK '52; or calico telescope-eyed goldfish) at a ratio of about (*TT*)1:(*Tt*)2:(*tt*)1. Before use, the homozygous nature of materials was ascertained by reciprocal crossing between two types.

The exchange of the eye was made reciprocally between two genetical strains *TT* and *tt*, in 2 to 3-day larvae after the differen-

tiation of the chromatophores, or in the tail-bud embryos before pigment-cell formation. In some cases embryos of different stages were used. In some other cases the eye was implanted subcutaneously between two types of different sizes.

For the exchange of the eye, embryos were anaesthetized in 1/10 saturated chloretone solution for ten to twenty minutes. After this, they were placed in pairs on filter paper in a shallow Petri dish, filled with double-strength HOLTFRETER's solution, and covered with a piece of filter paper except the head region to keep them immobile. The operation was made only on the right side, whereas the left eye remained intact as control. The skin was cut along the margin of the cornea with a fine glass or steel needle and the eyeball was extirpated by cutting eye muscles and the optic nerve. The eyeball was then implanted into the eyehole of the host in the normal orientation. It was not necessary to hold the transplanted eye in position for the healing of the wound. After the operation, the host animals were left anaesthetized for fourty-five to sixty minutes more by adding a few drops of 1/5 saturated chloretone solution. They were then transferred into double-strength HOLTFRETER's solution.

In the case of subcutaneous implantation, host animals were not anaesthetized. In the dorso-lateral part of the trunk, a few scales were removed and a small pocket in the dermis was made with a scalpel in which the extirpated eye was implanted in the way that the iris was directed to the surface.

The operation was made at room temperature, and the operated embryos were kept at 21°C. For histological investigations the ordinary double staining with hematoxylin and eosin was employed. To observe the guanine crystal layers CARNOY's fixative without acetic acid, and 1% toluidine-blue staining were adopted.

RESULTS

(*A*) *Normal development of the larval chromatophores in three different types of goldfish*

Homozygous normal type (*tt*): At room temperature of about 21°C., the outer layer of eye vesicles becomes pigmented following the onset of neurulation, within forty hours after spawning. In ten

hours, melanophores appear superficially in the anterior trunk region along the ventral ridge of the myotomes and the aorta in which blood-circuration has just begun. The line of melanophores differentiated soon spreads along the whole length of the ventral ridge of the myotomes. Another group of melanophores appears in the head and trunk regions. The tail fin remains free from melanophores. On the fourth day after spawning, the embryos begin to hatch and after hatching melanophores increase in number and spread over the entire dorsal surface.

Guanophores appear first on the eye about eighty hours after spawning. A few pale amoeboid cells, containing small guanine granules, are seen on the eyeball. During a few successive stages of development the cells increase in number, forming a continuous layer with reflecting guanine crystals, over the eyeball. Histologically the guanophore sheet takes the appearance of a twisted rope in section (Fig. 1).

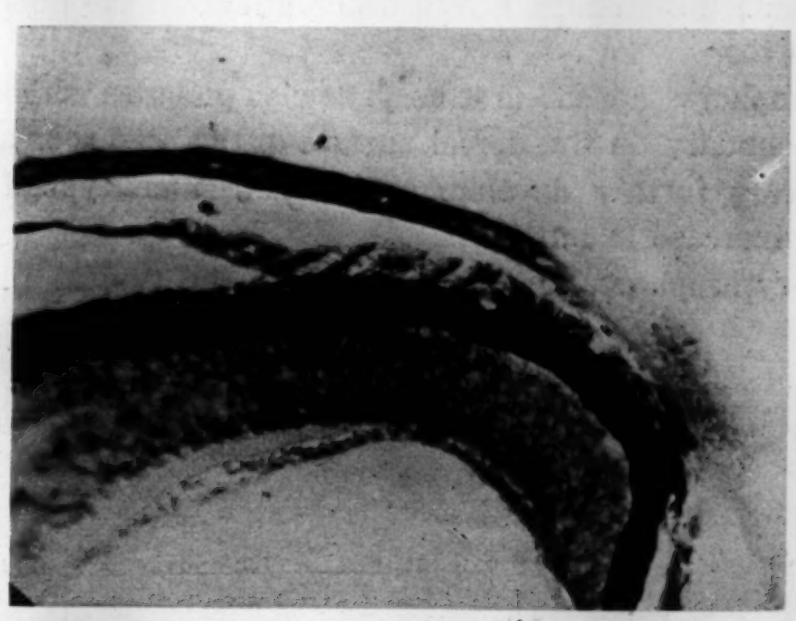

Fig. 1. Transverse section through the upper part of the iris in the fish of normal type. The guanophore sheet takes the appearance of a twisted rope.

About two days after hatching, guanophores become visible along the dorsal ridge of the endodermal duct, and soon they migrate to form a sheet around the peritoneum. Later on, about four weeks after hatching, when the larvae reached a length of more

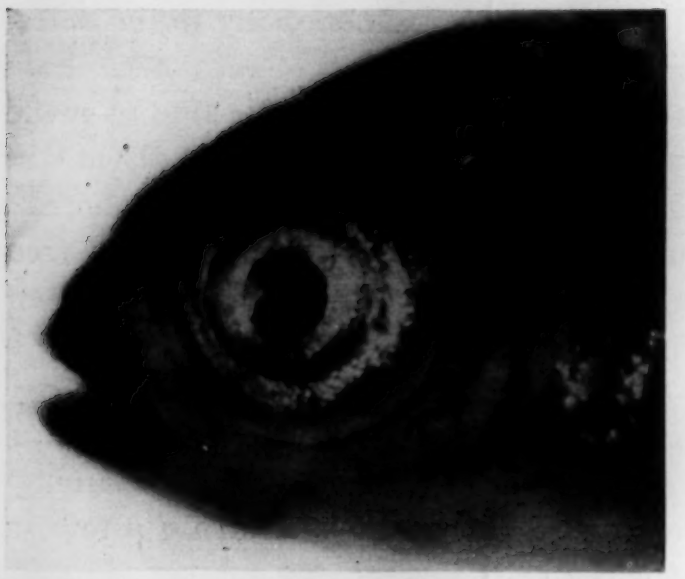

Fig. 2. The eye of the normal larva. The guanophores begin to differentiate in the operculum. 4 weeks after hatching.

than 12 mm., the guanine layers differentiate on the operculum and the scales too.

Xanthophores appear first as yellowish globules without showing cellular boundary on the dorsal surface of the head soon after hatching. During further development they assume typical amoeboid form and increase in number.

The erythrophores, giving the goldfish the typical fine colours,

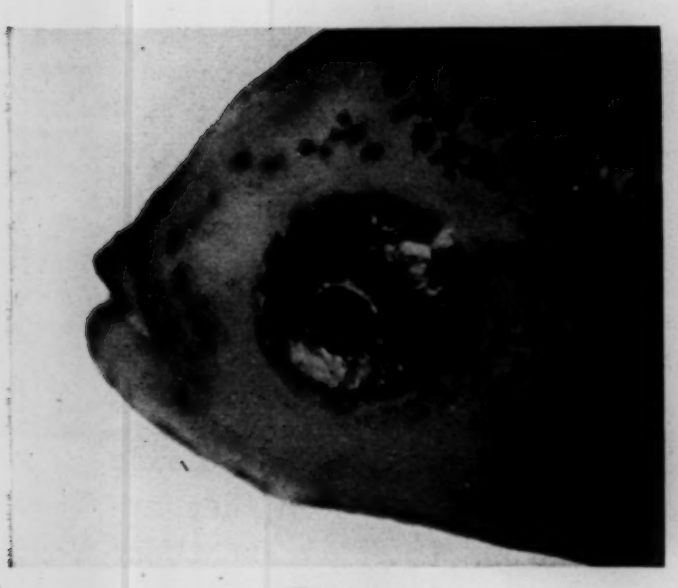

Fig. 3

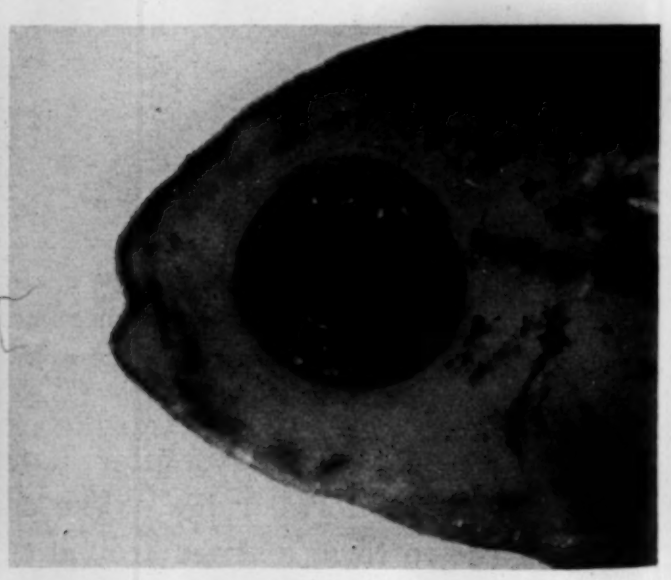

Fig. 4

appear only after the decolouration of the young fish, usually two to three months after hatching.

Homozygous transparent type (*TT*): Until about ten days after hatching, when the larvae have reached 6 to 7 mm., the homozygous transparent type cannot be distinguished in pigmentation from the normal type. Melanophores, xánthophores and guanophores differentiate in the same way as in the homozygous normal type, and they increase steadily in number. Ten days after hatching, the three kinds of chromatophores begin to depigment. After this critical stage both types can easily be distinguished by their different colours.

At first xanthophores along the dorsal trunk region begin to lose their pigment. The depigmentation extends forward and backward till the xanthophores completely disappear within a few days. The larva then becomes gray in colour.

Shortly after the depigmentation of xanthophores, guanophores of the eye begin to disappear and several black spots become visible on the iris surface (Fig. 4). These spots gradually spread till the whole iris becomes black. Almost at the same time guanophores on the dorsal surface of the choroid also begin to lose the reflecting granules so that the eyeball becomes almost entirely black (Fig. 5). The course of the disappearance of guanophores on the eye is plotted in Fig. 6. Guanophores of the peritoneum also disappear almost

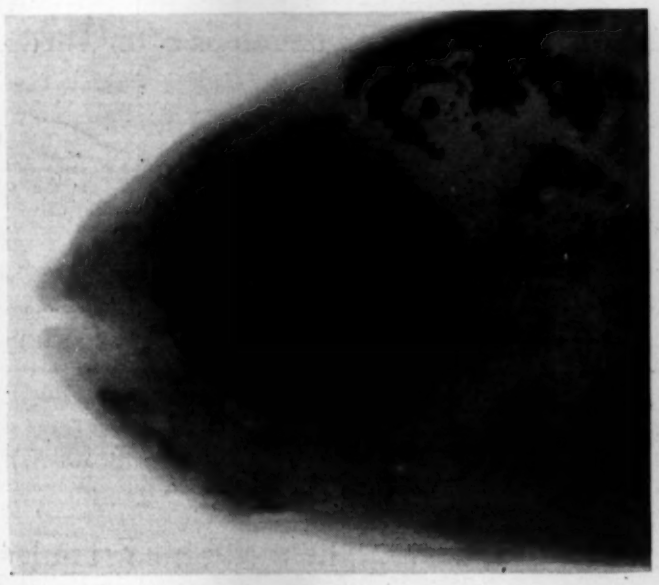

Fig. 5

Fig. 3-5. Degeneration process of eye-guanophores in the same fish of the transparent type.——Fig. 3. The eye of *TT* larva, just before the guanophore-degeneration. 10 days after hatching.——Fig. 4. 2-week *TT* larva. The eye-guanophores begin to disappear.——Fig. 5. 4-week *TT* larva. The eye-guanophores have completely disappeared. The melanophores in the head and operculum are degenerating.

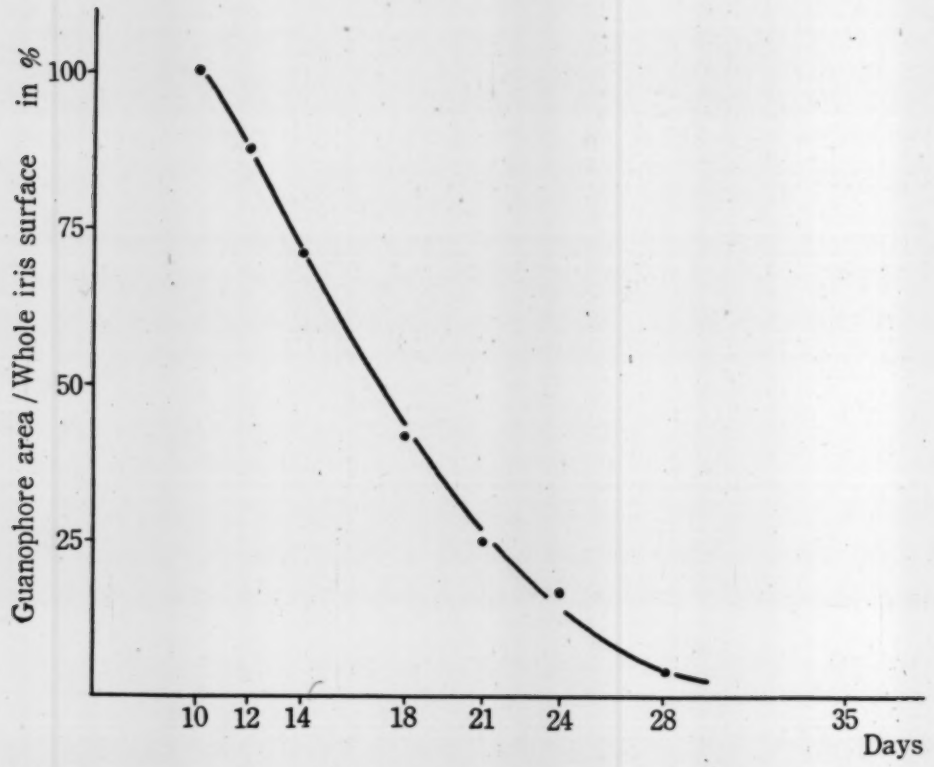

Fig. 6. The change of the area with guanophores on the iris of transparent type. The area with guanophores was drawn with a camera lucida, then it was cut and mesured by weighing. Each value is an average of ten specimens.

simultaneously.

Thus the reflecting guanine layers usually disappear completely in a short time in the homozygous transparent fish. In some cases, however, the guanophores are left in small areas on the peritoneum and the eye, as well as on the operculum which develops afterwards. An example is illustrated in Table 1.

Table 1. Behaviors of guanophores in three genic types, after the degeneration period of larval chromatophores

Genotype	Total number of cases	Phenotype			
		Guanophore lost	Guanophore retained		
			iris	peritoneum	operculum
TT	87	71(81%)	6(7%)	8(9%)	4(5%)
Tt	141	0(0%)	123(87%)	131(93%)	127(90%)
tt	89	0(0%)	89(100%)	89(100%)	89(100%)

Finally melanophores begin to disappear. The melanine granules first come together to form small globules, which remain in position

till they disappear in a few days. Ordinarily the depigmentation occurs throughout the entire body surface as the result of which the skin becomes quite transparent. In rare cases, however, melanophores remain in restricted areas on the head or/and at the junction of the caudal fin and caudal peduncle.

Heterozygous transparent type (*Tt*): The pigmentation of this type does not differ from that of the other two types until the larva reaches the length of 6 to 7 mm. After this stage the depigmentation of chromatophores proceeds on the body surface as in the homozygous transparent type. Generally in the heterozygous type the disappearance of chromatophores occurs only in the restricted area and it proceeds rather slowly. During the course of the chromatophore disappearance larvae show wide colour variations.

(*B*) *Transplantation and implantation of the eye*

(*a*) *Reciprocal transplantation on 2 to 3-day larvae*

At the time of operation, the outer surface of the eye, both in the normal and in the transparent type, was already covered with the reflecting guanine layer. The blood vessels of the iris could be clearly seen against the bright background. Two or three days after operation, the blood circulation in the vssels recovered in the majority of grafted eyes. If the eye had not badly been injured by the operation, it grew to the size corresponding to that of the host eye without losing its characteristic appearance. After about two months, however, a gradual reduction of the eye size became apparent, which was followed by degeneration of tissues. Although the eye usually degenerated within three months, in some cases it remained healthy for more than five months. In all cases the optic nerve of the transplanted eye formed a neuroma-like mass and none of its fibers grew back to join the brain.

1) *TT eye in TT larva*: The results are summarized in Table 2. About seven days after transplantation, xanthophores of the host skin began to depigment as had been observed in normal *TT* larvae. Next, the guanophores of the grafted eye began to disappear in the same rate as those of the host eye. Within four weeks the entire surface of the both eyes became black. Apparently the depigmentation is not due to the injury of the transplantation.

Table 2. Behaviors of guanophores after the reciporcal eye-transplantation between 2 to 3-day larvae

operation	Total number of cases	Behavior of guanophores			
		guanophore disappeared		guanophore retained	
		Host eye	Graft eye	Host eye	Graft eye
TT to *TT*	14	14	14	0	0
tt to *TT*	35	33	0	2	35
TT to *tt*	25	0	24	25	1
tt to *tt*	10	0	0	10	10

2) tt eye in TT larva: In thirty-five successful cases, the transplanted eye healed completely within twenty-four hours and blood circulation was recovered within two to three days. The growth of the transplanted eye was comparable with that of the control eye. At the depigmentation stage of the larval chromatophores of the host animals, however, the transplanted *tt* eye was retaining its reflecting tissue entirely, showing a clear contrast with the black eye of the host (Fig. 7). Only in two out of thirty-five cases disappearance of the reflecting layer of the host eye was not complete (Table 2). They probably belonged to the exceptional variation mentioned in the preceding chapter. At any rate, the reflecting layer of the transplanted eye remained healthy for more than two months till it began to degenerate. It is apparent from this result that the depigmentation of the guanine layer of the transplanted eye is not caused by the injury of the operation, but by the action of gene borne in the transplanted eye itself.

3) TT eye in tt larva: On the other hand, when the eye of the transparent fish was transplanted to the normal host, guanophores of the transplanted eye began to disappear at the time when the host had attained a length of 6 to 7 mm., and when the chromatophores of the host began to degenerate. Up to the stage, growth and pigmentation of the transplanted eye were not distinguishable from those of the control eye. After this stage, however, as the result of degeneration of the guanine layer, the surface of the grafted eye became mottled till it became entirely black (Fig. 8). Guanophores of the host, however, did not lose their refulgence either in the eye or on the entire body surface throughout the whole experimental period.

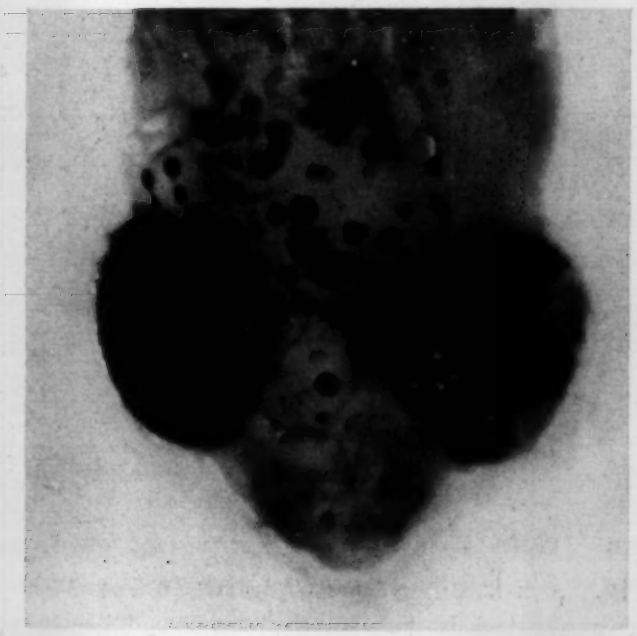

Fig. 7. Transplanted eye of the normal type on the *TT* fish. The guanophores of the transplanted eye remain unaffected, whereas those of the host eye have completely disappeared.

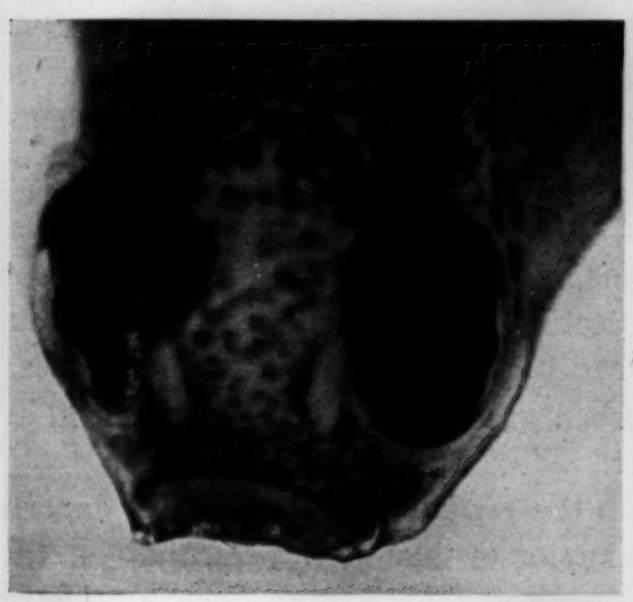

Fig. 8. The *TT* eye, transplanted before guanophore-degeneration stage on the normal fish. The guanophores of the transplanted eye disappeared completely, whereas the chromatophores of the host remained unaffected.

Fig. 9. Secondary guanophores in the *TT* eye transplanted in the normal host. The larval guanophores have once degenerated completely.

These results confirm the conclusion of the preceding experiment that the gene actions for the disappearance of larval guanophores in the transplanted eye have already been determined at the time of the operation.

However, the disappearance of guanophores in the transplanted *TT*-eye was only temporary. Within ten days the secondary guanophores began to appear and increased in number, spreading over the iris, till they covered the whole surface of the eye (Fig. 9).

4) *tt eye in tt larva:* When transplantation was performed between two normal types the grafted eye showed no sign of degeneration of its reflecting layer whatever. The wound healing and growth of the eye took place as in the other series, and the grafted eyes were resorbed gradually in the third month, even in the most successful cases.

Summing up the results of the fore-going experiments, it is obvious that the behavior of guanine layers, that is, their maintenance in the normal type and the disappearance in the transparent type, is governed by a gene which is borne in the eye itself.

(*b*) *Reciprocal transplantation of eye-anlage in neurulae*

Which tissue of the eye is responsible for the determination of the guanophore-behavior? To answer this question some experiments

were performed.

In teleostean embryos, although the origin of the larval chromatophores has been studied by several workers (LOPASHOV, '44; ORTON, '53; KAJISHIMA, '58), no experimental studies have been performed regarding the eye chromatophores. In amphibian embryos BARDEN ('42) studied the origin of eye chromatophores. He confirmed by transplantation experiments that xanthophores, guanophores and melanophores of the larval eye are formed from colourless chromatoblasts derived from the neural crest.

Embryos used for the present series were of about thirty to thirty-five hours after spawning. At this stage the eye-anlage was not yet pigmented. By unexpected operational difficulties, only eight cases out of sixty-seven transplantations were successful. In these cases, guanophores of the grafted eye differentiated in the pattern similar to that in the host eye. The experimental results are summarized in Table 3.

Table 3. Behaviors of guanophores after the reciprocal transplantation of eye-anlages between neurulae.

operation	Total number of cases	Behavior of guanophores			
		guanophre disappeared		guanophore retained	
		Host eye	Graft eye	Host eye	Graft eye
tt to *TT*	5	5	3	0	2
TT to *tt*	3	0	1	3	2

The eye of the normal type, transplanted to the transparent fish (*tt* to *TT*), acquired the normal pigmentation of the iris and the choroid at the beginning. At the depigmentation stage of the host, however, guanophores of the transplanted eye also began to disappear in the same way as in the host. In three out of five cases guanophores disappeared entirely from the eye surface and in the remaining two cases the degeneration of guanophores was incomplete, so that the eye showed mottled patterns (Table 3).

In the reverse transplantation (*TT* to *tt*) the grafted eyes of the transparent type retained their refulgence for more than two months in two cases in the normal host. In one case, some iris guanophores degenerated at an early stage.

These data are apparently contradictory to the preceding ex-

periments (Table 2), in which guanophores of transplanted eyes showed self differentiation. It seems probable that the difference in the outcome may be due to the difference in the origin of eye chromatophores. In the preceding cases, guanophores of the transplanted eye had apparently been formed already at the time of operation, while in the present experiments they were probably derived from the host and not provided by the donor. Therefore, it may be considered that eye guanophores of the goldfish are derived, as in amphibian eyes, from the other part of the embryos, probably from the neural crest or its equivalent.

If it is true, the characteristic behavior of the transparent type, which is manifested in the disappearance of larval guanophores is based only on the gene carried by guanophores themselves but not by other tissue of the eye.

(*c*) *Transplantation between larvae of different stages*

It still remains a question whether the disappearance of guanine granules is determined solely by the factors intrinsic to the guanophores, or there are other factors which are also responsible for disappearance?

To answer this, the eye transplantation was made between larvae of different developmental stages. As the host 10-day larvae and as the donor 2 to 3-day larvae were used. The diameter of the iris of the transplanted eye was about 0.4 mm. and that of the host eye was about 0.7 mm. The operation was rather easy, the wound being healed within a day, and circulation being resumed on the succeeding day. Since the growth of the transplanted younger eye was fast, it caught up the host eye in size within a month. The guanophore-behavior in the transplanted eyes after the guanophore-degeneration stage of the fish is summarized in Table 4.

In the transplantation of young eyes of the normal type in older larvae of the transparent type (*tt* to *TT*), the host began decolouration within a few days, but the transplanted eye retained its refulgence throughout the whole experimental period, although it seemed somewhat fainter than in the eye of the control. It was probably due to the lack of guanoblasts migration from the host.

On the other hand, guanophores in the eye of the transparent type, transplanted to the older fish of the normal type (*TT* to *tt*),

Table 4. Behaviors of guanophores in the eye transplanted between the different developmental stages.

operation	Total number of cases	Behavior of guanophores			
		guanophore disappeared		guanophore retained	
		Host eye	Graft eye	Host eye	Graft eye
tt to *TT*	8	8	0	0	8
TT to *tt*	17	0	16	17	1

began to depigment two to three days after operation (*i.e.* five to six-days after hatching), when the blood circulation in the grafted eye had been recovered, and the diameter of the iris was 0.55 mm. on the average. It became entirely black within a few days. As mentioned in the previous section, guanophores in the intact eye of the transparent type began to disappear usually about ten-days after hatching, when the diameter of the iris had reached more than 0.75 mm. It was shown by the present experiment that the disappearance of guanophores was remarkably accelerated in the transplanted eye as compared with the eye in the intact state.

From these results it will be concluded that eye-guanophores of the transparent type have inherent ability to decompose their guanine granules, but the time of decomposition is determined by the other factor. Eye-guanophores of the normal type, on the contrary, are devoid of such an ability to decompose guanine granules.

(*d*) *Subcutaneous implantation of the embryonic eye*

In this series of experiments the embryonic eye was implanted subcutaneously in the trunk region in the normal orientation. The grafts were prepared from 2 to 3-day larvae both of the homozygous transparent and of the normal types. As the host a three month larvae and adults of the transparent type alone were used. The host fish was entirely free from pigment cells, so that the implanted eye could be observed through the integument.

The blood circulation was not recovered in the iris of the implanted eye. The eye-guanophores first degenerated in all cases, even in the grafts of the normal type. However, there was difference in the time of degeneration between the two types of grafts (Table 5).

In the three month host, the guanophore-degeneration began on

Table 5. Beginning of guanophore-degeneration in the subcutaneously implanted larval eyes.

Operation	Total number	Beginning of guanophore-degeneration
TT to *TT* (3 months)	11	6.64±0.18 days
tt to *TT* (3 months)	10	7.40±0.17 days
TT to *TT* (adult)	9	7.22±0.15 days
tt to *TT* (adult)	6	7.33±0.17 days

the seventh or eighth day after implantation in the normal type, whereas in the transparent type it began on the sixth or seventh day. Duration of the degeneration till its completion was also different in both cases of experiments. In the normal type the degeneration took place more than ten days, whereas in the transparent type it was completed within ten days.

On the other hand, when adult animals were used as the hosts, no significant difference could be observed either in the time of beginning or in the length of time for the completion of guanophore-degeneration between the two types.

The experiments show that surrounding factors responsible for the guanophore-degeneration were still effective in the three month larvae, whereas in the adult fish they became inactive. Therefore, it is considered that the gene can be active during a certain limited period of development, beginning from about ten days after hatching, and ending at a time prior to maturation.

Discussion

In the previous paper (Kajishima, '58), in agreement with Orton ('53) and others, the neural crest origin of chromatophores in the teleostean embryos has been made probable, in conformity with the conclusions reached in amphibia, birds and mammals. As to the choroidal melanophores, Ris ('41) was the first to show the evidence of the neural crest origin in birds. Shortly after that Barden ('42) demonstrated the same origin of xanthophores and guanophores of the embryonic iris in amphibia. In the present paper it has been shown that the guanophores of the fish eye are derived extrinsically probably from the neural crest or its equivalent.

From the reciprocal transplantation of the larval eye and of the

eye-anlage, it has been concluded that the genetical characteristics of the guanophores in the transparent type have already been determined in the guanophores themselves at the time of operation. In the eye transplantation between different stages, it has been confirmed that the time of guanophore-degeneration is controlled by surrounding factor which is effective only from the tenth day after hatching till before maturation. It has also been found that guanophores of the transparent eye degenerate faster than in the intact condition. These results lead us to the conclusion that the guanophores of the transparent type are ready to reveal the gene action even before the surrounding factor becomes effective in the normal course of development.

These conclusions apparently differ from those obtained by DALTON ('49, '50) in the amphibian. He investigated the action of alleles controlling the development of the white (*dd*) and black (*DD* and *Dd*) pigment patterns in the Mexican axolotl. The results of the transplantation and of explantation *in vitro* of the neural crest have shown that the genetical differences in pigmentation lies in the difference in the tissue environment and not in the difference of the chromatophores themselves.

The gene effects of the transparent type differ from those controlling the eye pigments in some insect, in which the gene reveals its action through diffusible substances (cf. CASPARI, '49; NOLTE, '50–'59; KIKKAWA, '53).

There are some difference of opinion as to whether the scale transparency and the body pigmentation are controlled by the different genes. CHEN ('28) has concluded that the scale character and the body colour are determined by the same gene, because the duplex condition of gene *T* causes not only the entire or nearly entire degeneration of guanophores but also the depigmentation of melanophores and xanthophores at the same time.

On the other hand, MATSUI ('34) has concluded that the body colour is governed by different genes from those which govern disappearance of guanophores, because the homozygous transparent-scaled type sometimes produces mottled fish, whereas some heterozygous transparent fish produce no mottled offspring.

Recentry AFFLECK ('52) has expressed a similar opinion to MATSUI's that the actual pigmentation of nacreous (mottled) fish is

obviously controlled by genes other than those responsible for the amount of the guanine tissue. He investigated the distribution of chromatophores in three genic types obtained from the nacreous goldfish. He has found two different kinds of specimens in the homozygous transparent type. The one was very intense orange in colour and the other was mottled. From this finding he concluded that the differences between the three genic types, regarding the amount of reflecting tissue, are determined by one pair of alleles other than the genes responsible for the usual colouration.

In the course of the present study, some transparent specimens showed red patterns on their body surface. But erythrophores in these specimens were always formed secondarily after the disappearance of the larval (primary) chromatophores, more than two months after hatching. These results agree with those obtained by GOODRICH and HANSEN ('31), who have ascertained that larval melanophores and xanthophores always disappear before the secondary colouration. The red specimens noted by AFFLECK seem to be the same as those found by the present author, and their colouration must be due to the secondary colouration, which should be strictly distinguished from the primary (larval) pigmentation. In reality the distinction of the two kinds of pigment cell-formation were often overlooked.

The findings of GOODRICH and HANSEN however do not agree in detail with the present study. For instance, they concluded that the gene which governs the decolouration of larval melanophores acts at the time when the larvae have reached to the length of about 9 mm., whereas the gene under consideration controlling the disappearance of eye guanophores acts already in the prehatching stage. They have given no statement whether both the genes are identical or not.

It is necessary to make further experiments to obtain the critical evidence for deciding this question.

The author wishes to express his sincere thanks to Professor T. SATO for his constant encouragement and to Professor K. DAN for reading the manuscript. Thanks are also due to Dr. Y. MATSUI for his helpful criticism, and finally to Mr. M. KATO for his kind preparation of the photographs.

Summary

1. The racial character of the transparent-scaled type (*TT*) of the goldfish is imperfectly dominant to that of the normal type (*tt*). In the *TT* fish not only reflecting guanine layers but also the melanophores and xanthophores degenerate at an early developmental stage. In order to analyse the gene action in the degeneration of guanophores, transplantation and implantation of the eye were undertaken.

2. In the reciprocal exchange of the eye in 2 to 3-day larvae, guanophores of the transplanted *TT* eye began to disappear when the chromatophores of the control *TT* fish began to degenerate, even if the eye was grafted on the fish of the normal type. The *tt* eye did not lose its refulgence regardless of the host.

3. When the still colourless eye-anlage of the neurula was exchanged between the two types, the results were completely reversed. The *tt* eye transplanted into the *TT* host lost its refulgence when the host chromatophores disappeared. The *TT* eye retained its refulgence in the *tt* host throughout the whole experimental period.

4. From these experiments it is concluded that the genetical characteristics of this fish has already been determined in guanophores of larval eye.

5. In the eye transplantation between individuals of different stages, the transplanted *TT* eye-guanophores lost their refulgence sooner than the normal condition. This was taken to indicate that guanophores are ready to reveal their gene action at any time even before the time of the natural guanophore-degeneration, and the time to reveal their action is determined by the surrounding factor.

6. In the subcutaneous implantation of the embryonic eye in the 3-month larva, guanophores of the implanted *TT* eye began to degenerate sooner than those of *tt* eye. When an adult was used as the host, no significant difference could be found between the two types. It is apparent from these results that the surrounding factor responsible for the disappearance of guanophores is effective only during a limited period of development.

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Errata

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p. 286, line 4, for "E 0" read E 1

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